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Rsu-1 is a cDNA previously identified as an inhibitor of Ras-induced transformation. An HA-epitope tagged Rsu-1 cDNA was introduced into the MCF7 breast carcinoma cell line. Stable transfectants were selected for analysis of effects of Rsu-1 expression on growth control and Ras-dependent kinase pathways. Assessment of biological activity of HA-Rsu-1 transfectants revealed slower anchorage dependent growth rates , a significant reduction in anchorage independent growth and increased apoptosis compared to control MCF7 cell lines. Analysis of cell cycle regulatory proteins required for transit through G1 revealed that HA-Rsu-1 transfectant cell lines expressed elevated levels of $p21^{CIP}$ CDK inhibitor but not cyclin D or $p27^{KIP}$. This appears to be due to stabilization of P53 by Rsu-1 expression as revealed by pulse-chase experiments. Moreover, Rsu-1 transfectants exhibited reduced levels of cyclin D-associated kinase and cdk2 kinase activity. Perturbations in signal transduction pathways which can be activated by Ras were detected in the Ha-Rsu-1 transfectants including activation of Jun kinase, Rho kinase and c-myc transcription. Growth inhibition was not observed in Rsu-1 transfectants of the p53 mutant cell lines MDA-MB-231 and MDA-MB-468. Hence, Rsu-1 expression critically altered cell cycle regulation and growth of MCF7 cells as well as signaling pathways required for malignant growth.

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INTRODUCTION

Epidermal growth factor (EGF) family of ligands bind to a number of related receptors and stimulate mitogenesis in mammary cells. Signal transduction downstream of the EGF family of receptors involves activation of the GTPase Ras and its effectors. Expression cloning to isolate inhibitors of Ras-induced transformation identified the Rsu-1 cDNA [4]. Previous experiments demonstrated that the expression of the Rsu-1 gene in NIH3T3 fibroblasts under the control of a heterologous promoter could suppress transformation by v-Ras but not v-Raf, v-Mos, or v-Src oncogenes [4], and experiments in our laboratory have determined that the Rsu-1 suppressor inhibits EGF- and TGFa-induced anchorage independent growth of NOG8 mouse mammary cells. *RSU-1* is a highly conserved single copy gene which encodes a protein of 33kD. p33 Rsu-1 contains a series of 23 amino acid leucine-based amphipathic repeats homologous to the repeats found in yeast adenylyl cyclase in the region through which Ras activates adenylyl cyclase in *Sa. cerevisiae* [4]. This suggested that Rsu-1 might interact with Ras or Ras-regulatory or -effector molecules. Transfection of a Rsu-1 vector into NIH3T3 cells and PC12 cells resulted in an increase in signal to Raf-1 and Erk-2 kinase and a decrease in signal transduction to Jun kinase [8]. These results demonstrate that Rsu-1 can alter signal transduction downstream of Ras.

Signal transduction "downstream" of Ras depends on the association of Ras with its effector proteins. Several proteins have been identified which associate with Ras in a GTP-dependent manner. These include Raf-1, RasGAP, p110 subunit of PI-3-kinase, Rin-1, Mek kinase 1, protein kinase C zeta, and RalGDS; in the case of several of these proteins (i.e. Raf-1, RasGAP, p110 PI3 kinase) signal transduction pathways activated as a result of interaction with Ras have been characterized. Activation of Ras effectors can lead to phosphorylation and activation of kinases, cytoskeletal proteins and transcription factors. For example, activation of Raf-1 results in specific phosphorylation of Mek and Erk-2 [14], and use of dominant negative Erk demonstrated that inhibition of this pathway interefered with Ras transformation in fibroblasts [3]. The small G proteins Rac and Rho are activated independently of Raf-1 by an as yet uncharacterized mechanism in response to activated Ras, and very recent evidence suggests that they play a crucial role in growth and tumorigenesis of epithelial cells. Studies using dominant negative Rho and Rac indicated that inhibition of Rho and/or Rac pathways prevented transformation by Ras, suggesting that these proteins regulate pathways essential for Ras transformation [6, 12, 13]. Activated Rac and cdc42 induced transformation in epithelial cells [7], whereas activation of the Raf-1/Erk pathway alone was not sufficient to transform rat intestinal and MCF10A epithelial cell lines [10]. These results, along with studies describing properties of transformants produced by specific effector mutants of Ras [1, 7, 15], have led to the conclusion that several pathways "downstream" of Ras contribute to

transformation of cells in response to activated Ras. Most interestingly, activation of Rho and Rac appears to be responsible for induction of anchorage independent growth and tumorigenicity in epithelial cells [7]. Because cdc42Hs and Rac have been shown to be required for the activation of the stress-activated or Jun kinase [2, 9, 11], and RhoA has been shown to be necessary for activation of the SRF by serum and other agents that act through the pertussis-sensitive G protein pathway [5], it appears that the small G proteins may exert their effects via transcriptional activation. Because pathways dependent on Rac and Rho are important for maintaining the transformed state, inhibitors of this pathway should prove especially useful in further dissection of growth control pathways and in developing antitumor therapies. Because the Rsu-1 suppressor has been shown to inhibit transformation and events dependent on Rac and Rho [8] we are investigating its role in the growth regulation of breast carcinoma.

The experiments reported here were designed to assess the ability of Rsu-1 to regulate pathways "downstream" of Ras which are activated by members of the EGF receptor family in human mammary carcinoma cells. Rsu-1 transfectants have been prepared in the MCF 7 cell line for analysis of response to EGF and estrogen. The Rsu-1 transfectants have been compared to vector control transfectants for anchorage-dependent and -independent growth. The effect of Rsu-1 on the Ras-induced activation of downstream effectors (RasGAP, Raf-1, Mek kinase, and PI3-kinase) has been determined by analyzing the kinases regulated by specific Ras effectors following stimulation of the cells. The kinases include: MAP kinases Erk-2, Jun kinase, p38 kinase, and Akt kinase (protein kinase B), αPak, and Rho kinase. The effect of Rsu-1 expression on activation of myc transcription has been examined. The influence of estrogen on the regulation of the EGF-dependent pathways in Rsu-1 transfectants of MCF7 cells has been tested. In these experiments using a luciferase reporter gene under the control of estrogen responsive promoter elements no effect of Rsu-1 was detected. The analysis of cell cycle alterations in Rsu-1 transfectants revealed that Rsu-1 expression results in stabilization of p53 and increased levels of the protein, the accumulation of $p21^{CIP}$ and arrest of cells in the G1 phase of the cell cycle. Transfectants in the MDA-MB468 cells are being evaluated for effects of Rsu-1 on EGF-dependent signaling. The difficulties using vectors for the constitutive expression of Rsu-1 in MCF10A cells will be described along with a description of the inducible vector system now being employed.

BODY:

TECHNICAL OBJECTIVE 1. Construction of Rsu-1 and vector control transfectant cell lines.

Several vectors have been used for the expression of p33Rsu-1 in human breast epithelial cell lines. These vectors include: p3v36 which contains the *RSU-1* cDNA under the control of a MT-1 promoter in a retroviral vector [4], p3v65 which contains an HA-tagged version of the *RSU-1* cDNA under the control of a RSV promoter in a vector containing an SV40 origin of replication [8], and p3v64 which contains an HA-tagged *RSU-1* cDNA in a retroviral vector under the control of a MT-1 promoter. MCF 7 and MCF10A cell lines have been transfected or infected with the above vectors and the appropriate "empty" control vectors. Following selection in G418 single colonies have been isolated using cloning cylinders, expanded into cell lines and screened for *RSU-1* RNA expression. In the case of transfectants containing the p3v36 or p3v64 vectors the transfectants were assayed in the presence and absence of Cd++ which results in induction of transcription from the MT-1 promoter. The following table lists transfectants prepared to date.

MCF 7	p1521	control vector	transfectants obtained
MCF7	p3v36	*Rsu-1* vector	transfectants obtained
MCF7	p521	control vector	transfectants obtained
MCF7	p3v65	*Rsu-1* vector	transfectants obtained
MCF10A	p1521	control vector	transfectants obtained
MCF10A	p3v36	*Rsu-1* vector	transfectants obtained*
MCF10A	p3v64	*Rsu-1* vector	transfectants obtained*
MCF10A	p521	control vector	transfectants obtained
MCF10A	p3v65	*Rsu-1* vector	transfectants not obtained
MDA-MB-468	p521	control vector	transfectants obtained
MDA-MB-468	p3V65	*Rsu-1* vector	transfectants obtained

* No clones expressed Rsu-1 in response to Cd++.

It is interesting to note that the transfection of MCF10A cells with vector encoding *RSU-1* cDNA under the control of a constitutive promoter did not result in the isolation of any G418 resistant colonies. It appears that the growth suppressive properties of Rsu-1 are sufficient to inhibit the growth of normal cells expressing this protein at high level. MCF10A clones derived from infection of cells with a retroviral vector containing Rsu-1 under the control of an inducible MT-1 promoter have been isolated, but no clones express vector-derived RNA in response to Cd++ . Hence, another inducible system, the ecdysone responsive promotor vector, is being used (see below).

<u>MCF10A TRANSFECTANTS USING ECDYSONE-INDUCIBLE PROMOTOR VECTORS.</u>

As described above the transfection of MCF10A cells has not yielded transfectants which express HA-Rsu-1 constitutively or in response to Cd++ induced transcription from the MT-1 promoter. Clones of MCF10A have been obtained readily with the control (empty) vectors, and clones which contain vectors carrying the HA-Rsu-1 cDNA have been isolated by both transfection and retroviral infection. The latter clones, >20 in number, do not activate transcription of HA-Rsu-1 in response to Cd++. Because we have had success with the MT-1 promotor vectors in MCF10A cells for the expression of TGFalpha and c-ErbB2, it appears that the current problems are related to the expression of a growth inhibitory cDNA. Therefore, we have initiated use of the ecdysone-inducible promotor vector in these cells. Ecdysone is an insect steroid hormone which recognizes a receptor not found in any mammalian cells [18]. The first step in the use of this system was the construction of cell lines expressing the two subunits of the ecdysone receptor, one of which has been engineered to contain the DNA binding domain of VP16. The sequences encoding the two subunits of the receptor are contained in the vector, pVgRXR (commercially obtained from In Vitrogen, Carlsbad CA). Together these subunits serve to activate transcription in the promotor of the expression vector, pIND, in response to muristerone A/ ponasterone. The HA-Rsu-1 cDNA has been introduced into the pIND vector. The resulting plasmid, p3V77, has been functionally tested in NIH3T3EcR cells which contain the pVgRXR vector. Clones of MCF10A expressing the receptor have been isolated and have been transfected with plasmids containing the ecdysone-responsive promotors. However, screening for inducible clones expressing HA-Rsu-1 did not reveal any cell lines in which HA-Rsu-1 expression could be induced and maintained.

MDA-MB-468 retroviral infectants have been isolated and biological experiments involving these clones are underway. Retroviral infection was used because transfection of this cell line was an inefficient method to introduce cDNA into the cells. The retroviral vector used was pLNSX.

TECHNICAL OBJECTIVE 2. Determine anchorage dependent and independent growth properties of selected transfectants.

MCF 7 TRANSFECTANTS

Transfectants in the MCF 7 cell line were analyzed for the effect of *Rsu-1* expression on biological properties of the cells and the results are described in the attached publication "Ectopic expression of Rsu-1 results in elevation of $p21^{CIP}$ and inhibits anchorage independent growth of MCF7 breast cancer cells" [19]. The studies described in figures 1, 2 and 3 of this publication include the effect of Rsu-1 on anchorage-dependent and -independent growth.

Experiments on the Rsu-1 and control infectants of the MDA-MB 468 cell line are described in figure 2 in the appendix. Expression of Rsu-1 inhibited anchorage dependent and independent growth of the cells.

TECHNICAL OBJECTIVE 3. Determine the ability of transfectant cell lines to form tumors in athymic mice.

These studies have been initiated using the MDA-MB-468 transfectants but have not revealed differences between control and transfectant cell lines. This may be due in part to the ability of a small number of these highly tumorigenic cells which either express lower levels of Rsu-1 or cease expression of Rsu-1 following injection to expand and produce a tumor. The analysis of expression levels in the cells should reveal if this is the case.

TECHNICAL OBJECTIVE 4. Analysis of the Ras signal transduction pathway in response to growth factor in transfectants.

The effect of Rsu-1 expression on the activation of the Ras signal transduction pathway has been examined and the results are described in the attached publication [19] "Ectopic expression of Rsu-1 results in elevation of $p21^{CIP}$ and inhibits anchorage independent growth of MCF7 breast cancer cells". Treatment of control and transfectant cell lines with growth factor, serum or TPA was used to activate Ras. Then the activation of specific kinases which are dependent on specific Ras effectors was tested in control as well as HA-Rsu-1 expressing clones. Erk, Jun kinase, Rho kinase and Pak were analyzed in control and Rsu-1 transfectant cell lines. The results can be seen in figures 4 and 5 of appendix reference 19.

Regulation of Myc transcription in Rsu-1 transfectants (figure 1). Our previous studies had indicated that the expression of Rsu-1 inhibited the level of myc transcription in response to serum and growth factor [figure 6, reference 19]. The induction of myc transcription following stimulation with serum, EGF or TPA for 1 hour in MCF7 and Rsu-1 MCF7 transfectants was determined in more detail by RT-PCR. Induction of myc RNA by serum and growth factor was reduced in the Rsu-1 transfectants compared to the control cells. This result supports our previous findings obtained by Northern blotting [19]. However, the data in figure 1 of this report indicate that induction of myc expression by TPA was enhanced by Rsu-1 expression in MCF7 cells. Recent studies demonstrated the stabilization of myc protein by Ras expression [17] and the regulation of myc RNA stability by a RasGAP binding protein [16]. These studies suggest that additional examination of the mechanism of regulation of myc RNA levels in Rsu-1 transfectants would be useful.

Increased levels of $p21^{CIP}$ and p53 in Rsu-1 transfectants. Following the determination that $p21^{CIP}$ levels were elevated by Rsu-1 expression in the PC12 cell line [20] we examined the effect of Rsu-1 expression on cell cycle regulation in MCF 7 cell transfectants. Rsu-1 expression resulted in an increase in $p21^{CIP}$ but no change in cyclin D1 expression [reference 19, figure 7]. However, cyclin D-associated kinase and cdc2 kinase activities were reduced in Rsu-1 transfectants compared to control cells [19]. Further investigation revealed that the mechanism of increased $p21^{CIP}$ expression is likely to be an increase in stability of the tumor suppressor p53, which transcriptionally regulates $p21^{CIP}$ expression (attached reference 21 figure 1). The MCF 7 cell line contains a wild type or non-mutated version of p53 which is normally found at very low levels in the cell. Rsu-1 expression resulted in a elevation of p53 protein and pulse chase

experiments revealed that p53 was more stable in Rsu-1 transfectants (attached reference 21, figure 2).

Activation of Ras-dependent pathways in the absence of estrogen. The effect of estrogen on the stimulation of Ras-dependent pathways in the MCF7 and Rsu-1 MCF7 transfectants has been tested. The experiments performed include the comparison of activation of Ras-dependent kinase pathways in the presence and absence of estrogen. Cells were grown to near confluence then incubated in phenol red-free media containing 1% charcoal stripped serum in the presence or absence of estradiol. No change has been observed in the activation of Erk, Jun kinase or Rho kinase in the absence of estrogen in MCF 7 cell Rsu-1-transfectants following growth factor stimulation. No differences in the activation of the early response gene transcription in the presence and absence of estrogen has been observed. Finally, no change in the induction of luciferase reporter activity was detected upon transfection of a plasmid containing luciferase under the transcriptional control estrogen responsive elements into Rsu-1 and control MCF 7 cell transfectants.

Activation of Ras-dependent pathways in MDA-MB-468 cells. Because this cell line contains high number of EGF receptors treatment of control and infectant cell lines with EGF was used to activate Ras. Then the activation of specific kinases which are dependent on specific Ras effectors was tested in control as well as HA-Rsu-1 expressing clones. Erk, Jun kinase, Rho kinase and Pak were analyzed in control and Rsu-1 infectant cell lines. The results of some of these assays can be seen in appendix figure 2. There was inhibition of activation of Jun kinase by EGF and this effect was most discernable change in signal transduction pathways. The other observable difference between vector control and Rsu-1 infectant cell lines was in the level of $p21^{CIP}$. However, there was little change in p53 levels in these cells which are p53 mutant. This suggests that the change in $p21^{CIP}$ detected in this cell line may be independent of p53.

TECHNICAL OBJECTIVE 5. Analyze transphosphorylation and hetero-dimerization of EGFfamily receptors in response to growth factor.
These experiments were performed in the MDA-MB-468 Rsu-1 transfectants. However, to date no differences have been detected between Rsu-1 and vector control cell lines.

TECHNICAL OBJECTIVE 6. Characterize Rsu-1 binding proteins in human breast cancer cell lines.
These studies have not yielded definitive information on the identity of Rsu-1 binding protein(s). Studies ongoing in our laboratory are aimed at identification of Rsu-1 binding proteins using yeast two-hybrid screening. Results of these studies will be applied to MCF7 cells.

Identify p33Rsu-1 *in vivo* binding partners:
While there is considerable evidence for disruption of the Ras pathway by Rsu-1, it is not clear that Ras (Ha-, Ki- or N-Ras) is the normal p33Rsu-1 *in vivo* binding partner. Also, the carboxy terminus of Rsu-1 does not contain the leucine rich amphipathic repeat sequence proposed as a binding domain for Ras. Therefore, it is likely that this region of

the protein binds to a different protein. Identification of the leucine repeat and carboxy-terminal protein binding partners, would provide valuable information on the role of Rsu-1 in Ras signaling. The original co-immunoprecipitation experiments proposed in the application yielded no informative data. No co-immunoprecipitation has been detected with signaling proteins on the Ras pathway. Therefore, it appeared that identification of a binding protein by co-immunoprecipitation would require scale-up of the precipitaions and then identification of bands by MALDI-TOF or microsequencing methods. The alternative to this approach is to use the yeast two hybrid system to clone a binding protein. We have chosen the latter apprach. While this is beyond the scale of the experiments proposed in the original application the screening is underway in our laboratory.

The yeast dual-hybrid system is a well established method for the detection of *in vivo* binding of proteins. A positive signal in this assay for protein binding partners relies on transcriptional activation of selectable markers and reporter genes as a result of interaction of two proteins, one a fusion with an activation domain (AD) and the other a fusion with a DNA binding domain (BD). The system we initially used for our studies relies on GAL4 binding to upstream activator sequence (UAS) DNA and activation of transcription of nutritional markers. Specific regions of the Rsu-1 protein (referred to as "bait") were introduced into a vector with the open reading frame fused to a GAL4 DNA binding domain. The cDNA library to be screened contains cDNA clones fused to the GAL4 activation domain. Human brain libraries have been chosen because, while Rsu-1 expression is ubiquitous, brain is a tissue in which Rsu-1 is expressed at a high level. By plating yeast co-transfected with the Rsu-1 binding domain vector and the cDNA activating domain library on nutritionally defined media, yeast which have activated transcription of genes required for survival on the deficient media, due to interaction between BD and AD fusion proteins, can be selected. The yeast selected in this way were screened using several different nutritional selection conditions, to confirm the presence of both vectors and activation of the GAL4-dependent reporters. The vector systems to be used allow selection using three or four nutritional markers to reduce the number of false positive clones and to allow for selection of "weak" or "strong" interactions. Subsequent to the selection of candidate interacting clones, the clones have been recovered and retested in nutritional and beta-galactosidase assays prior to DNA sequencing of the cDNA inserts.

Several different Rsu-1 GAL4 vectors have been prepared fusing separate regions of the Rsu-1 protein to GAL4 binding domain. The amino terminal two-thirds of the Rsu-1 molecule, which contains the leucine repeat region, has been cloned "in frame" with the GAL4 DNA binding domain. In addition, the entire Rsu-1 open reading frame has been cloned into a vector for GAL4 DNA binding domain fusion protein expression. Each of these constructs has been used for library screening. In all cases the vectors were transfected into host strains and tested for toxicity during liquid growth of the yeast by comparison to control vectors. The yeast expressing the fusion protein were tested for background transcriptional activation using growth on selective media.

To date > 5,000,000 clones have been screened to date using this method; >1500 HIS, LEU, TRP colonies have been picked and replica plated. >100 "true" positive library

clones have been isolated, tested for beta-galactosidase activity, recovered from yeast and are being retested for binding activity. Following retransfection into yeast and a repeat of the nutritional selection and beta-galactosidase assays, candidiates have been chosen for sequencing and further analysis. DNA sequencing was performed with vector derived primers to allow determination of cDNA insert sequence and reading frame. Sequences have been searched for identity to known genes by Blast search. To date the clones expressing some degree of positivity encode ferritin-L, a member of the COP9 signalasome recognition particle, and IKB. Experiments will continue to identify a Rsu-1 binding protein by this method.

TECHNICAL OBJECTIVE 7. Test ability of Rsu-1 to suppress transformation induced by Rho and Rac proteins.

It has not been possible to carry out the experiments proposed in tasks 11 and 12 because a Rsu-1-MCF10A transfectant cell line has not been obtained. Hence, the approach described of measuring the effect of Rsu-1 expression on ROCK activation has served as our method of determining the effect of Rsu-1 on the Rho pathway.

TECHNICAL OBJECTIVE 8. Determine the effect of Rsu-1 mutation on its ability to inhibit anchorage independent growth.

Rsu-1 mutants have been constructed and transfected into MCF7 cells. Truncation of Rsu-1 at the carboxy terminus results in unstable protein products. Carboxy terminal truncations of Rsu-1 were produced with an HA epitope tag at the 5' end and introduced into expression vectors. These clones were transfected into Cos1 cells and tested for level of protein produced. No protein was detectable by Western blotting at 48 hours post transfection whereas there was abundant protein in cells transfeted with HA-tagged full length Rsu-1. Pulse chase experiments revealed that the half life of the truncated proteins was less that one hour whereas that of the full length HA-Rsu-1 was greater that 6 hours. Rsu-1 is serine phosphorylated in response to growth factor (G. Dougherty and M.L. Cutler, unpublished observation) and mutants of the main phosphorylation site (serine 163) were prepared (ser163ala) and tested for growth inhibiting activity in MCF 7 cells. While MCF7 cells expressing this mutant inhibited the growth of MCF 7 cells, mutation at this site abrogated the ability to enhance apoptosis. These results are included in Figure 3 and Table I in the appendix.

Individual clones of MCF7 cells expressing Rsu-1 mutants have been tested for anchorage-dependent and -independent growth, expression of cell cycle proteins and apoptosis. Appendix figures 3 and Table I.

TECHNICAL OBJECTIVE 9. Screen human breast cancer cell lines for Rsu-1 RNA and protein (figure 3).

RNA and protein have been tested for Rsu-1 expression by Northern blotting and Western blotting. The cell lines tested include: MCF10A, MDA-MB-468, MDA-MB-231, ZR-75-1, A1N4. The results indicate that while all cell lines contain ample RNA the level of endogenous Rsu-1 protein is not as high in breast carcinoma or non-transformed mammary epithelial cells (MCF10A) as in fibroblasts. The results are shown in figure 4 in the appendix.

TECHNICAL OBJECTIVE 10. Analyze Rsu-1 expression in human breast tumors.
task 17. Perform SSCP on DNA or RNA RT-PCR samples from human breast tumor using Rsu-1 primers. We have changed our approach to this objective. A new monoclonal antibody to Rsu-1 has been developed and we will use the antibody for immunohistochemistry to stain section of breast tumors. We stated in the proposal that these studies would be performed if time and resources permit. They are not complete at this time.

KEY RESEARCH ACCOMPLISHMENTS:

-demonstration that Rsu-1 negatively regulates anchorage-dependent and -independent growth of MCF7 breast cancer cells
-demonstration that Rsu-1 expression inhibits cell cycle progression by stabilization of p53 with consequent increase in $p21^{CIP}$ cdk inhibitor and cell cycle arrest
-demonstration that Rsu-1 expression enhances apoptosis in p53 positive MCF7 breast cancer cells
-demonstration that Rsu-1 expression inhibits Jun kinase and Rho kinase activities and prevents growth factor, but not TPA, activation of c-myc transcription
-demonstration that Rsu-1 expression does not affect estrogen regulation of breast cancer cell growth
-demonstration that mutation of a major site of phosphorylation in Rsu-1 protein alters its ability to enhance apoptosis, but not the ability to inhibit growth.

REPORTABLE OUTCOMES:

Three publications (two published, one submitted)

1. Vasaturo, F., Dougherty, G, and M.L. Cutler. 2000. Ectopic expression of Rsu-1 results in elevation of p21CIP and inhibts anchorage-independent growth of MCF7 breast cancer cells. Breast Cancer Research and Treatment. 61:69-78.

2. Masuelli, L, Ettenberg, S, Vasaturo, F, Vestergaard-Sykes, K and M.L. Cutler. 1999. The Ras suppressor Rsu-1 enhances nerve growth factor-induced differentiation of PC12 cells and induces $p21^{CIP}$ expression. Cell Growth and Differentiation 10:555-564.

3. Vasaturo, F., Chopp, T., and M.L. Cutler. 2001. Expression of Rsu-1 results in p53-dependent growth inhibition in fibroblasts and enhances apoptosis in MCF7 cells. Oncogene (submitted).

CONCLUSIONS:

Rsu-1 transfectants have been successfully prepared in two cell lines, MCF7 and MCF10A. The expression of Rsu-1 effects the anchorage-dependent growth rate in MCF7 breast carcinoma cell line, and Rsu-1 inhibits anchorage-independent growth. Analysis of the influence of Rsu-1 expression on Ras-dependent pathways has indicated that there is an increase in the activation of Erk 2 in response to EGF and TPA. There is a decrease in the activation of Jun kinase in response to serum and EGF, but little change is detected in the PI-3-kinase dependent activation of AKT kinase in respnse to serum and EGF. However, activation of Rho alpha kinase by EGF is inhibited in the transfectants, but there is little effect on the activity of the Rac and Cdc42 dependent αPak serine threonine kinase by Rsu-1 expression. The results indicate that the expression of Rsu-1 does influence signal transduction "downstream" of Ras. In particular, there is inhibition of Rho dependent signaling events. These transfectants will allow the continued delineation of the role of various Ras effectors in the activation of downstream kinases in human mammary carcinoma cell lines. Correlation of biochemical changes to inhibition of specific biological properties should point to the specific signal transduction pathway(s) responsible. At present the role of Rsu-1 in altering signal transduction in the Ras pathway in response to growth factor and serum has been tested.

Our experiments have demonstrated that the influence of estrogen on alteration of Ras pathway by Rsu-1 is not significant. Further experiments to determine the effect of the number of EGF receptors, dimerization of receptors and, hence, the level of activation of the Ras pathway, on the influence of *Rsu-1* on downstream kinase pathways have not revealed clear differences between high and low EGF receptor cell lines. While there is growth inhibition in both breast cancer cell lines, the changes in the signal transduction pathways tested are more apparent in the MCF7 background.

The most exciting discovery about the role of Rsu-1 in regulation of signal transduction has been the demonstration that Rsu-1 transfectants have elevated levels of the cdk inhibitor $p21^{CIP}$ and that this elevation is probably due to an elevation in p53 tumor suppressor protein[19,20, 21]. P53 elevation appears to result from increased p53 stabilization in Rsu-1 transfectant cell lines. Most importantly, the stabilization of p53 appears to contribute to an increase in apoptosis in the MCF7-Rsu-1 transfectants. Future studies are aimed at understanding the nature and mechanism of the p53 stabilization and the increase in apoptosis. Moreover, the MDA-MB-468 Rsu-1 transfectants will be used to determine the effect of Rsu-1 on EGF-induced apoptosis in this cell background. Since Rsu-1 expression inhibits some effects of EGF-induced signal transduction in this background, these studies should reveal the details of the nature of the signals leading to apoptosis in these cells.

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Three publications (two published, one submitted)

1. Vasaturo, F., Dougherty, G, and M.L. Cutler. 2000. Ectopic expression of Rsu-1 results in elevation of p21CIP and inhibts anchorage-independent growth of MCF7 breast cancer cells. Breast Cancer Research and Treatment. 61:69-78.

2. Masuelli, L, Ettenberg, S, Vasaturo, F, Vestergaard-Sykes, K and M.L. Cutler. 1999. The Ras suppressor Rsu-1 enhances nerve growth factor-induced differentiation of PC12 cells and induces $p21^{CIP}$ expression. Cell Growth and Differentiation 10:555-564.

3. Vasaturo, F., Chopp, T., and M.L. Cutler. 2001. Expression of Rsu-1 results in p53-dependent growth inhibition in fibroblasts and enhances apoptosis in MCF7 cells. Oncogene (submitted).

ABSTRACTS:

1. Vasaturo, F. and M.L. Cutler. 2000. Ectopic expression of Rsu-1 results in elevation of $p21^{CIP}$ and inhibts anchorage-independent growth of MCF7 breast cancer cells. Annual Meeting of the American Association for Cancer Research. San Francisco, CA

2. Vasaturo, F. and M.L. Cutler. 1999. Ectopic expression of Rsu-1 results inhibts anchorage-independent growth of MCF7 breast cancer cells. Annual Meeting of the Society for Cell Biology, Washington, D.C.

3. Vasaturo, F. and M.L. Cutler. 2000. Ectopic expression of Rsu-1 results in elevation of p21CIP and inhibts anchorage-independent growth of MCF7 breast cancer cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting. Atlanta, GA

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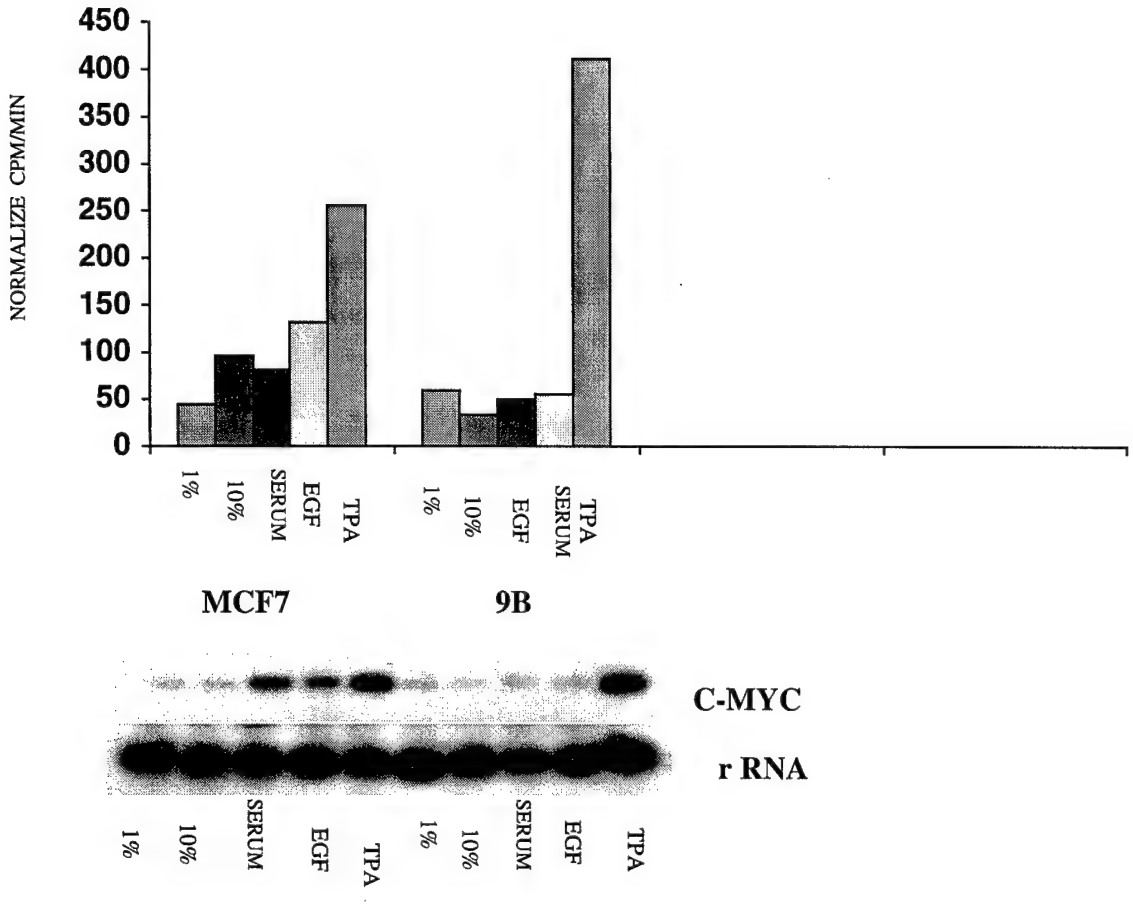
Suryapraba Chunduru, Ph.D.

Appendix

FIGURE 1

RSU-1 EXPRESSION INHIBITS C-MYC TRANSCRIPTION IN MCF7 CELLS



The expression of c-myc RNA in response to serum, EGF and TPA stimulation was analyzed in MCF7 HA-Rsu-1 transfectants. Cells were propagated in low serum for 16 hours and then stimulated with serum (20%) , EGF (200ng/ml) and TPA (200 ng/ml) for 1 hour. RNA was isolated and used for quantitative RT-PCR using ribosomal RNA amplification as a normalization control. Induction of c-myc RNA was reduced approximately in 50% in the HA-Rsu-1 transfectants 1 hour post serum addition, and 70% post EGF addition compared to the control cells. However, there was no block in the ability of TPA stimulation to induce c-myc transcription in Ha-Rsu-1 transfectants. These results suggested that Rsu-1 had inhibitory effect on growth factor-induced activation of c-myc expression.

Figure 2a.

MDA-MB-468 cell lines expressing HA-Rsu-1proteins were seeded for anchorage independent growth in 0.3% agar media. Three plates of 10,000 cells per plate were seeded for each clone. The MDA-MB-468 pLNSX vector control cell line was included. The data represents the total number of colonies scored after 14 days of growth in agar. The total number of colonies per plate (average of three plates) is indicated.

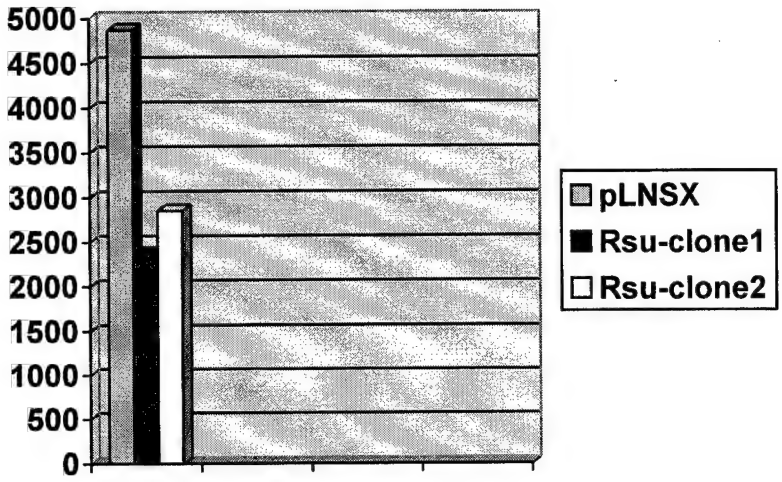
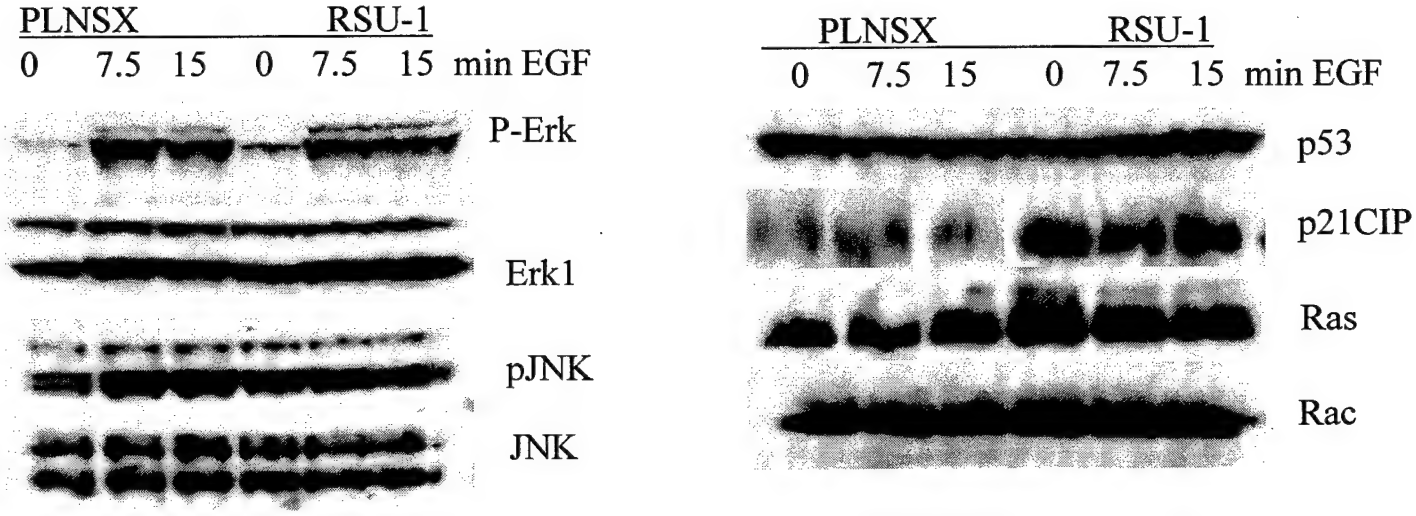


Figure 2b



Western blots of lysates from PLNSX or Rsu-1 vector infectants of MDA-MB-468 cells. Cells were serum starved overnight then exposed to EGF (100ng/ml) for the designated time. Lysates were prepared, separated on 10 or 12% SDS PAGE gels and used for Western Blots. Blots were reacted with the indicated antibodies and then stripped and reacted sequentially with additional antibodies.

Figure 3

Rsu-1 mutants S4A and S163G retain MCF7 growth inhibitory properties

MCF7 transfectant cell lines expressing Rsu-1 phosphorylation mutant proteins were seeded for anchorage independent growth in 0.3% agar media. Three plates of 10,000 cells per plate were seeded for each clone. As the positive control the MCF7-14B cell line, which expresses wild type Rsu-1, was included. The data represents the total number of colonies scored after 14 days of growth in agar. Colonies of seven or more cells were scored as positive and a total of 100 grids (20% of the plate) was counted for each determination and extrapolated. The total number of colonies per plate (average of three plates) is indicated.

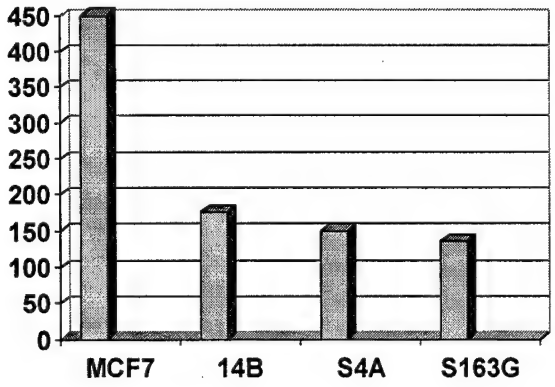


Figure 4

Expression of Rsu1 RNA in breast tumor cell lines.

Total RNA was extracted from human breast tumor cell lines using guanidine thiocyanate and purified using CsTFA centrifugation. Ten micrograms of the recovered RNA was separated on 1% agarose gels, transferred to a nylon membrane and hybridized to a ^{32}P-labeled probe for human Rsu-1 and then GPDH. All breast tumor cell lines contain three RSU-1 RNA species (4kb, 2.2kb, and 1.8kb) while the human fibroblast cells contain only the 4 kb and 1.8 kb RNAs.

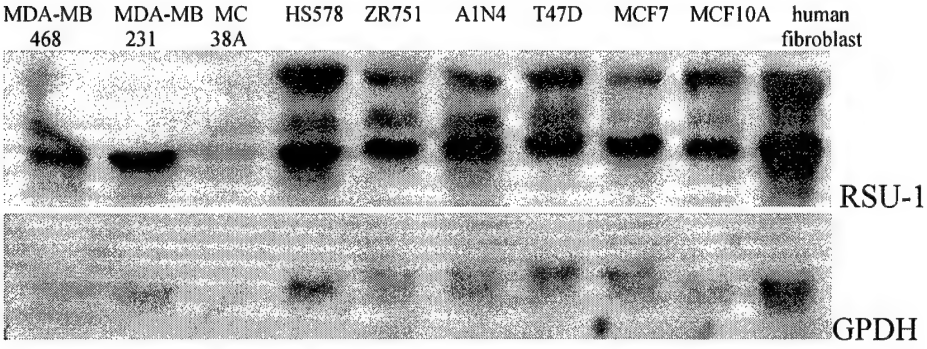


Table 1

Cell line	Treatment [a]	% apoptotic cells
MCF7-control	(-)	< 0.1
	Stauro 12 hours	41
	Stauro 16 hours	47
	TNFα+CHX 12 hours	26
	TNFα+CHX 16 hours	34
MCF7-Rsu-1-9B	(-)	< 0.1
	Stauro 12 hours	58
	Stauro 16 hours	70
	TNFα+CHX 12 hours	32
	TNFα+CHX 16 hours	44
MCF7-Rsu-1-S4A	(-)	< 0.1
	Stauro 12 hours	32
	Stauro 16 hours	45
	TNFα+CHX 12 hours	28
	TNFα+CHX 16 hours	38
MCF7-Rsu-1- S163G	(-)	< 0.1
	Stauro 12 hours	36
	Stauro 16 hours	39
	TNFα+CHX 12 hours	25
	TNFα+CHX 16 hours	35

5×10^5 cells were seeded into 60 mm plates 48 hours prior to treatment. A combination of TNFα (15ng/ml) and cyclohexamide (CHX) (10μg/ml) or staurosporine (1μM) was added to the cells in complete growth media. Initially the development of apoptosis was observed microscopically; nuclear condensation and membrane blebbing preceded cell rounding in treated cells. Apoptosis was scored in this experiment by trypan blue staining of treated and untreated cultures. Detached cells were combined with the trypsinized cell population at specific time points after treatment. Each point represents the average of three plates. The presence of nuclear condensation closely corresponded to trypan blue uptake.

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Ectopic expression of *Rsu-1* results in elevation of $p21^{CIP}$ and inhibits anchorage-independent growth of MCF7 breast cancer cells

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Key words: agar growth, Erk, Jun kinase, MCF7, $p21^{WAF/CIP}$, Rho kinase, Rsu-1

Summary

Signal transduction from tyrosine kinase receptors mediates growth regulation of breast cancer cells in part through the GTPase Ras and downstream kinases. Rsu-1 is a cDNA previously identified as an inhibitor of Ras-induced transformation. An HA-epitope tagged Rsu-1 cDNA was introduced into the MCF7 breast carcinoma cell line. Stable transfectants were selected and used for analysis of *Rsu-1* expression on growth control and Ras-dependent kinase pathways. Assessment of biological activity of HA-Rsu-1 transfectants revealed that HA-Rsu-1 clones showed slower anchorage dependent growth rates than control MCF7 cell lines and a significant reduction in anchorage independent growth. Analysis of cell cycle regulatory proteins required for transit through G1 revealed that HA-Rsu-1 transfectant cell lines expressed elevated levels of $p21^{CIP}$ CDK inhibitor. Perturbations in signal transduction pathways which can be activated by Ras were detected in the Ha-Rsu-1 transfectants. Exposure of serum-starved cells to EGF revealed that expression of *HA-Rsu-1* increased ERK-2 kinase activation, decreased activation of Jun kinase and inhibited Rho-dependent Rho-alpha kinase (ROK) activity compared to control cells. While serum starvation reduced AKT activity to undetectable levels in HA-Rsu-1 transfectants but not in control MCF7 cells, activation of AKT kinase by serum was unaffected by *HA-Rsu-1* expression. Finally, the level of c-myc transcription in HA-Rsu-1 transfectants reached only 60% of the MCF7 control cell line following serum stimulation of starved cells while Fos RNA levels were similar to control cells. These results demonstrate that increased *Rsu-1* expression critically altered cell cycle regulation and growth of MCF7 cells as well as signaling pathways in MCF7 cells required for malignant growth.

Introduction

Signals from numerous extracellular receptors including the estrogen receptor, the epidermal growth factor (EGF) receptor family, IGF-1 receptor and TGF-B receptor contribute to the growth regulation of breast cancer cells. Signal transduction downstream of the EGF family of receptors involves activation of the Ras GTPase and its effectors [7, 8, 28]. Although a number of experimental observations demonstrate that aberrant Ras function can promote malignant transformation of breast epithelial cells, mutated ras genes are infrequently seen in human breast carcinomas. However, the frequent amplification and overexpression of the Her2 / Neu/ Erb2 receptor tyrosine kinase, which in turn causes chronic activation of Ras and the Ras signal transduction pathway, suggest that deregulated Ras pathway signaling may be a common alteration in breast carcinomas [5, 10, 12, 20, 25]. Prominent phenotypic changes result from introduction of activated Ras into mammary epithelial cells, including unlimited cell division potential, rapid proliferation, and loss of adhesion requirement for survival and growth [2, 4, 41], and similar changes are seen as a result of *ErbB2* expression.

In this paper we study the role of Rsu-1, identified as an inhibitor of Ras-induced transformation, in cell cycle regulation and in the EGF signaling pathway in the estrogen dependent MCF7 breast carcinoma cell line. Rsu-1 is a highly conserved, ubiquitously

expressed, single copy gene which encodes a protein of 277 amino acids. Interestingly, the amino terminal two-thirds of p33 Rsu -1 is composed of a series of leucine-based repeats (LRR region) which share homology to yeast adenylyl cyclase in the region required for activation by Ras in *Saccharomyces cerevisiae* [14]. Previous experiments demonstrated that the expression of *Rsu-1* gene in NIH3T3 fibroblasts, under the control of a heterologous promoter, could suppress transformation by v-Ras but not v-Raf, v-Mos, or v-Src oncogenes, and ectopic expression of *Rsu-1* in NIH3T3 cells and PC12 cells resulted in an increase in Ras-dependent signal to RafB kinase and ERK-2 kinase [30] and a decrease in signal transduction to Jun kinase [31]. Therefore, in this study the effect of *Rsu-1* expression on biological parameters of growth and signal transduction pathways associated with Ras activation have been analyzed using Rsu-1 transfectants of MCF7 cells.

Studies describing properties of transformants produced by specific effector mutants of Ras [1, 23, 43], have led to the conclusion that several pathways 'downstream' of Ras contribute to transformation of cells in response to activated Ras. The small G proteins Rac and Rho are activated by an incompletely described mechanism in response to activated Ras, and recent evidence suggests that Rho and Rac appear to be responsible for induction of anchorage independent growth and tumorigenicity in epithelial cells in response to Ras [22, 23, 43]. Cdc42 and Rac have been shown to be required for the activation of the stress-activated or Jun kinase [13, 32, 35], and the activation of Jun kinase has been demonstrated to be essential for transformation [11]. Recent studies have demonstrated that Rho is required for SRE activation [19]. Studies using dominant negative Rho and Rac indicated that inhibition of Rho and /or Rac pathways prevented transformation by Ras, suggesting that these proteins regulate pathways essential for Ras transformation [22, 37, 38]. This conclusion was supported by the demonstration that activated Rac and Cdc42 induced transformation in epithelial cells [23], whereas activation of the Raf-1/Erk pathway alone was not sufficient to transform rat intestinal and MCF10 epithelial cells [34]. The Rsu-1 suppressor has been shown to inhibit Ras transformation and events dependent on Rho [30]. Therefore, we proposed to examine the role of Rsu-1 in the regulation of these pathways in breast carcinoma cell lines.

Materials and methods

Cell cultures

MCF7 cells were mantained in essential modified eagle medium (EMEM) with 10% fetal bovine serum (FBS), 2 mM glutamine, non essential amino acids and antibiotics. The transfectants clones were maintained in media supplemented with 200 μg/ml G418 sulfate.

Plasmid construction and transfection

The Influenza virus hemagglutinin epitope tag (HA-tag) was added to the amino terminus of the Rsu-1 open reading frame in a two-step PCR reaction. The HA tagged cDNA was introduced into p521 vector [30] under the transcriptional control of the *RSV* promoter and the resulting plasmid is referred as 3V65. MCF7 cell line was transfected with the above vector and the appropriate 'empty' control vectors using lipofectin reagent DOTAP (Boehinger Mannhem). Following selection in G418 single colonies were isolated using cloning cylinders, expanded into cell lines and screened for *RSU-1* RNA expression.

Northern blotting

Eighty percent confluent cell cultures were lysed with 1 ml of TriPure Isolation Reagent and RNA was isolated according to manufacturer's directions (Boehringer Mannheim). For some experiments RNA was isolated using CsTFA. Ten micrograms of total RNA was electrophoresed on a 1% agarose gel containing morpholinepropanesulfonic acid (MOPS; pH 7) and formaldhehyde (2%) and transferred to nylon transfer membrane (Nytran Plus) (Schleicher & Schuell). The membranes were hybridized with specific random primed probes as described previously [30].

Western blotting

Lysates (100 μg) were separated by SDS-PAGE, transferred to PVDF membrane and blocked overnight in 1× blocking reagent, 2.5% non fat dry milk, in Tris-buffered saline. The filters were reacted with the different primary antibodies: HA clone 12CA5 (Boehringer Mannheim), pan Ras (Oncogene Science), Rac 1, $p27^{KIP}$ (Transduction Laboratories), Ras Gap, RhoA and $p21^{CIP}$ (Santa Cruz Biotechnology), and cyclinD1 (Upstate Biotechnology). Filters were washed in Tris-buffered saline containing 0.4% Tween

20 (Sigma), and incubated with specific horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science Inc., Arlington Heights). The filters were developed by enhanced chemiluminescence, (ECL) using Kodak X-Omat film.

Anchorage-dependent and independent growth assays

MCF7 and clones were plated at 50,000 cells per 60 mm tissue culture dish in EMEM with 10% FBS. The cells were counted after 24, 48, 72, 96, 120 h. The number of cells reported at each time point was the average of two plates. For the soft agar cell growth, cells were plated in top agar (0.3%) in EMEM in 60-mm gridded tissue culture plates (Nunc Brand Products), over a bottom layer of 2 ml EMEM cointaining 0.6% agar. Plates were incubated for 10–14 days in a 5% CO_2 atmosphere at 37°C. Colonies larger than 60 μm were scored as positive. The number reported was the average of two plates, and the data was reported as the percentage of cells plated which formed colonies.

Protein kinase assays

Cell lysates were immunoprecipitated with specific antibody and protein A sepharose on ice for 1 h, washed extensively and incubated in kinase buffer containing γ -^{32}P- ATP and the appropriate substrate for 20 min at 30°C. The labeled substrates were resolved by SDS-PAGE and transferred to PVDF membrane. The filters were quantitated using a Packard Beta Scanner and exposed to film for autoradiography. Conditions for ERK and Jun kinase assays were as described previously [30]. For Pak kinase assay, the immunoprecipitates were recovered from 600 μg of lysates using an antiPaK (c-19) antibody (Santa Cruz Biotechnology). The buffer used for Pak kinase activity contained 10 mM Hepes, 1 mM $MnCl_2$, 0.1 M Sodium Vanadate, 20 mM $MgCl_2$, 25 mM β-glycerophosphate, 2 mM DTT, 1 μCi of γ -^{32}P- ATP and 5 μg of the target protein, Myelin Basic Protein (MBP).

For Rok α kinase assay, the immunoprecipitates were collected from 400 μg of lysates with antiRok$_\alpha$ antibody (Transduction Laboratories) and assayed in 30 μl of kinase buffer containing 10 mM HEPES, 10 mM $MnCl_2$, 2 mM $MgCl_2$, 1 μCi of ^{32}P-γ-ATP, and 5 μg Histone H1. The cyclin D-associated kinase activity and the cdk2 kinase activity were tested in immune kinase assays using antibodies for cyclin D (Upsate Biologicals) and cdk2 (Santa Cruz) and GST-Rb (Santa Cruz) as a substrate.

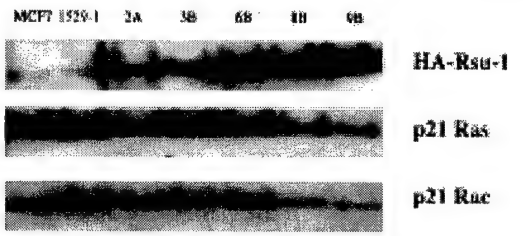


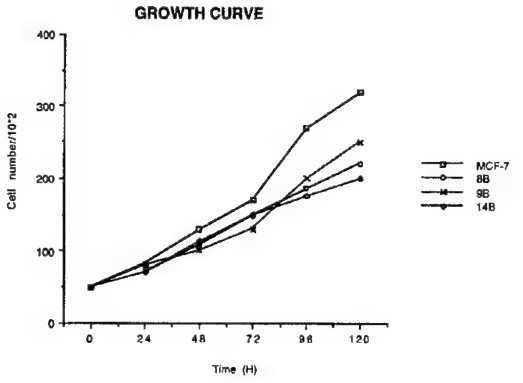
Figure 1. Expression of HA-Rsu-1, Ras and Rac proteins in MCF7 transfectant cell lines. MC7 cells were transfected with the HA-Rsu-1 vector p3v65 (clones designated as 2A, 3B, 6B, 8B and 9B) or the control vector p521 (clone 1529-1). Cell lysates were analyzed by western blotting for the expression of *p33 HA-Rsu-1* using a mouse monoclonal antibody directed against the HA epitope tag (clone 12CA5). Expression of *p21 Ras* was detected using a mouse monoclonal antibody which detects Ha-, Ki-, and N-Ras species (OP22). p21 Rac was also detected using a mouse monoclonal antibody for Rac. The PVDF filters were stripped and probed with each antibody sequentially.

Results

Construction of HA-Rsu-1 transfectant cell lines

An expression vector (p3v65) encoding HA-tagged Rsu-1 was transfected into the MCF7 cell line, which expresses a low number of EGF receptors and is a model of estrogen receptor (ER) positive breast cancer. The MCF7 cell line was also transfected with the 'empty' control vector, p521. Following selection in G418, single colonies were isolated using cloning cylinders, expanded into cell lines and screened for HA-Rsu-1 RNA and protein expression.

Total RNA from individual clones was analyzed by northern blotting using a probe specific for the vector-encoded murine Rsu-1 ORF. At high stringency this probe primarily detects the murine HA-Rsu-1 ORF and hybridizes poorly with endogenous human Rsu-1, which is expressed ubiquitously in MCF7 cells in culture. The results indicated that most clones contained several HA-Rsu-1 specific RNA species (data not shown). Clones containing HA-Rsu-1 specific RNA of predicted size were chosen for further study. Cell lysates were analyzed for expression of p33 HA-Rsu-1 by western blotting using a mouse monoclonal antibody specific for the HA epitope tag (clone 12CA5). The results shown in Figure 1 indicate that p33 HA-Rsu-1 was detected in all transfectants except 3v65-2A, a clone which had predominantly larger sized HA-Rsu-1 RNA. In addition, the levels of p33 HA-Rsu-1 were high in several clones, particularly 3v65-6B, 3v65-8B, and 3v65-9B (Figure 1). Expression of $p21^{Ras}$ and



CELL LINE	DOUBLING TIME	R^2
MCF7	43.2 h	0.968
8B	67.2h	0.994
9B	60 h	0.954
14B	74 h	0.993

Figure 2. Anchorage dependent growth of HA-Rsu-1 transfectants. HA-Rsu-1 transfectant and control cell lines were plated at 50,000 cells per 60 mm tissue culture dish in EMEM with 10% FBS. The cells were counted at 24, 48, 72, 96 and 120 h. The number of cells calculated at each time point was the average of two plates. All data represent the average of four independent experiments. Growth rate was determined by calculating the doubling time of each cell line from its growth curve.

$p21^{Rac}$ were detected in both transfectant and control cell lysates.

Anchorage dependent and independent growth of HA-Rsu-1 transfectants

Anchorage dependent growth of MCF7 cells expressing *HA-Rsu-1* was compared with the MCF7 control cell line. A growth curve was derived for each cell line by plotting the number of cells counted at 24 h intervals. The value for each point is the average of four determinations. The growth rate was determined by calculating the doubling time of each cell line from its growth curve. The results (Figure 2) indicate that the growth rates of HA-Rsu-1 expressing clones were 25–50% slower than the control cell lines.

Anchorage independent growth of HA-Rsu-1 transfectant and control cell lines was tested by seeding cells in soft agar. The percentage of control MCF7 cells giving rise to colonies was determined and normalized to 100%. Data for the HA-Rsu-1 transfectant colony growth were expressed as the percent of the MCF7 control cell line. The results (Figure 3) demonstrated that the HA-Rsu-1 transfectant cell lines exhibited less efficient growth in agar than the control MCF7 cell lines. In addition, the colonies produced by HA-Rsu-1 transfectants were smaller than those of

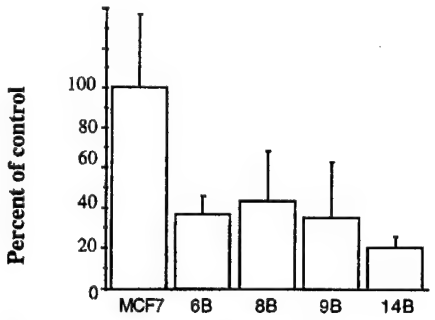


Figure 3. Anchorage independent growth of HA-Rsu-1 transfectants. HA-Rsu-1 transfectant clones 6B, 8B, 9B, 14B and control cell lines were seeded in 0.3% agar. Colonies were counted at 14 days post plating. The results of four separate experiments were averaged and the percentage of MCF7 cells giving rise to colonies was normalized to 100%. The values for the HA-Rsu-1 cell lines are expressed as the percent of the MCF7 control value. The number of colonies reported for each experiment was the average of two plates. Standard error values are indicated by bars – differences between transfectant and control clones were compared by analysis of variance followed by Scheffe's and Fisher's multiple sample tests ($P < 0.001$).

the control. It should be noted that the level of p33 *HA-Rsu-1* expression in transfectants correlated with a reduced efficiency of anchorage independent growth. This suggests that the changes in anchorage independent growth result from Rsu-1 induced changes in the cells. Also, the inhibition of colony formation by the Rsu-1 transfectant cell lines could not be reversed by inclusion of EGF or TGF-β in the agar (data not shown).

The effect of HA-Rsu-1 *expression on the activation of Ras-dependent signal transduction pathway*

To assay for defects in Ras effector function the activation of Ras-dependent kinases was tested in control as well as HA-Rsu-1 expressing clones following stimulation of cells with EGF, serum or TPA. The activation of Erk2 was determined by immune kinase assay in serum starved HA-Rsu-1 clones and control cell lines. Cells were stimulated with EGF (100 ng/ml) for 7.5 min. The results (Figure 4A) indicated that in MCF7 control cells and HA-Rsu-1 transfectants Erk2 was activated. However, there was a statistically significant enhancement in Erk2 activation in response to EGF in all HA-Rsu-1 transfectant clones compared to control. This is in agreement with results obtained in other cell types [30]. Erk2 activation by EGF in both control and Rsu-1 transfectant cells was inhibited by 1 h pretreatment with PD98058, an inhibitor of

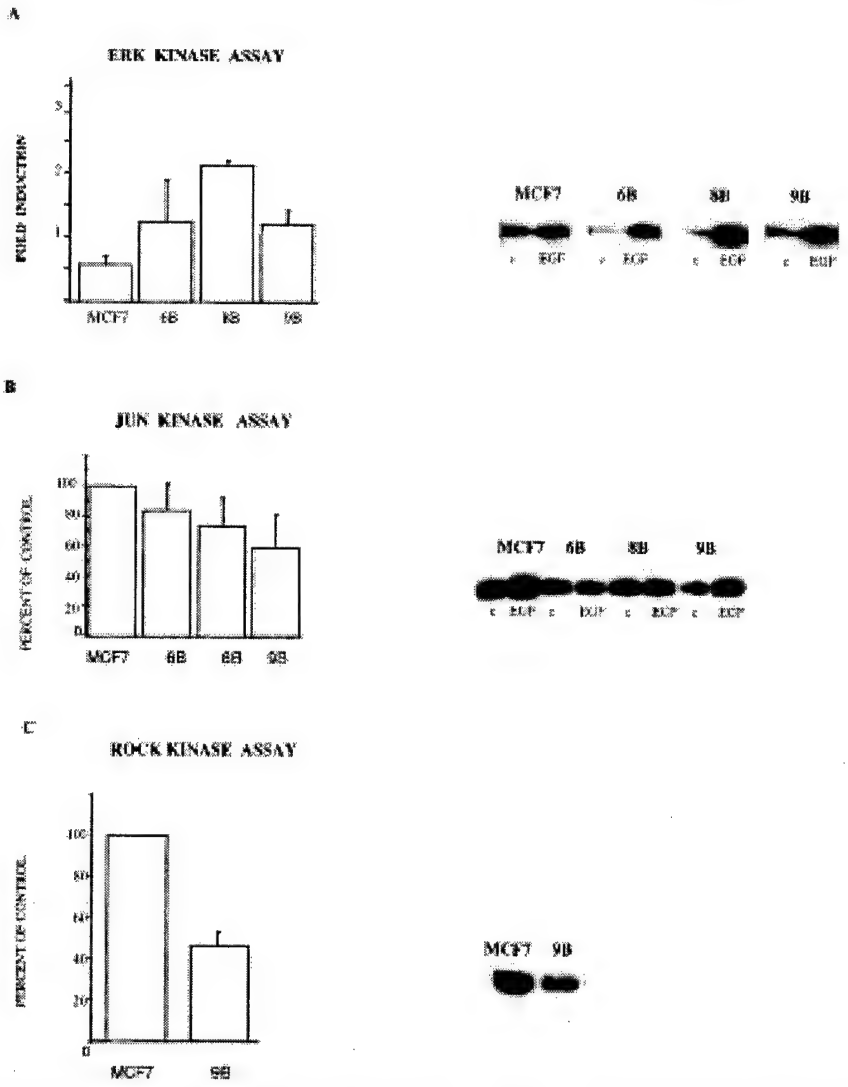


Figure 4. Activity of Erk2, JNK and Rho kinase in HA-Rsu-1 transfectants. A. HA-Rsu-1 transfectant and control cell lines were tested for Erk2 activity following EGF stimulation of serum starved cells. Cell lysates were prepared from cells treated with EGF (100 ng/ml) for 7.5 min and from untreated control cells. Erk2 was immunoprecipitated and the immune kinase activity was measured using MBP as a substrate. The fold-induction was measured and standard error values are indicated by bars. The data were compared by analysis of variance followed by Scheffe's and Fisher's multiple sample tests ($P < 0.01$). B. HA-Rsu-1 transfectant and control cell lines were tested for JNK activity following EGF stimulation for 20 min. c-Jun was immunoprecipitated and the kinase activity was measured using GST-ATF2 as substrate. The data represents the average of four experiments. C. HA-Rsu-1 transfectant and control cell lines were tested for endogenous Rho kinase (ROK-α) activity. ROK-α was immunoprecipitated and the kinase activity measured using Histone H1 as a substrate. The data represent the average of four experiments and the data were compared by analysis of variance followed by Scheffe's and Fisher's multiple sample tests ($P < 0.001$).

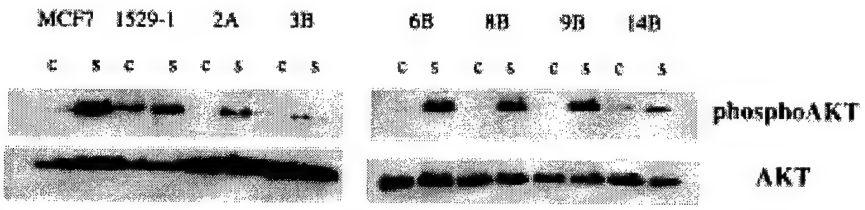


Figure 5. AKT Kinase activation of HA-Rsu-1 transfectant and control cell lines. AKT kinase activity was activated in serum starved cells by the addition of 20% serum to the cultures. The activation of AKT was measured 60 min after the addition of serum by western blotting of total cell protein with an antibody that detects phosphorylated AKT, followed by stripping and reprobing the filter with an antibody that detects both the phosphorylated and non-phosphorylated version of AKT kinase.

Mek (data not shown). An increase in Erk2 activation in response to TPA was also detected in HA-Rsu-1 transfectants relative to the control cells (data not shown).

Jun kinase (JNK) activation was also measured by immune kinase assay following EGF stimulation for 20 min (100 ng/ml). The results (Figure 4B) indicated that serum-starved HA-Rsu-1 clones exhibited lower JNK activity in comparison to the MCF7 control cell line. In addition, the amount of Jun kinase activity following EGF stimulation was less in the Rsu-1 transfectants than in the control cells (Figure 4B). Other data (not shown) suggested that the JNK activity in these cells resulted from expression and activation of both p54 and p46 Jun kinase. This inhibition of JNK activity is in agreement with results reported by us previously [30, 27]. We also compared activation of Erk and JNK pathways in cells grown in estrogen-depleted media to those grown in complete media. However, these experiments did not reveal significant qualitative and quantitative differences between the two conditions (data not shown).

Activation of the Rho signal transduction pathway was tested using an assay for ROK-α activity. ROK-α (RhoA kinase) is a 180 kd serine threonine kinase that interacts with the GTP-binding form of RhoA GTPase, and its activation correlates with the transforming functions of Rho [26]. ROK-α activation was tested in serum starved HA-Rsu-1 clones and control cell lines. Our results indicated that endogenous Rho kinase activity was present in the MCF7 cell line and this activity was reduced in HA-Rsu-1 transfectants (Figure 4C). Stimulation of cells with EGF did not significantly enhance this activity in either the MCF7 control or Rsu-1 transfectant cell lines (data not shown).

AKT kinase activity is dependent on the Ras effector phosphatidylinositol-3 kinase (PI-3 kinase). We tested AKT kinase activity in HA-Rsu-1 transfectant and control cell lines by addition of serum to quiescent, serum-starved cultures. AKT activation was measured 60 min post addition of serum by western blotting of total cell protein, using an antibody specific for the Ser 473 phosphorylated version of AKT kinase [17]. This was followed by stripping and reprobing the filter with an antibody which detects the nonphosphorylated form of AKT kinase. The results (Figure 5) demonstrated that following serum starvation, HA-Rsu-1 expressing clones showed nondetectable levels of phosphorylated AKT kinase, while some residual level of phosphorylated AKT was seen in control cells. In response to serum addition, phosphorylation of AKT kinase was detected in both control cells and HA-Rsu-1 transfectants. These results suggested that *Rsu-1* expression did not completely disrupt activation of either Ras-dependent PI3-kinase or AKT kinase.

The activation of the serine threonine kinase α-Pak, the Rac p21 activated kinase, was also tested by immune kinase assay. Serum starved HA-Rsu-1 and control cell lines were stimulated with either TPA or EGF. The results indicated that the Rac and Cdc42 dependent α Pak activity was not altered by *HA-Rsu-1* expression (data not shown).

Expression of c-myc *in HA-Rsu-1 transfectants*

The expression of *c-myc* RNA in response to serum stimulation was analyzed in MCF7 HA-Rsu-1 transfectants. Cells were serum starved for 16 h and then stimulated with 20% serum for 1 h. RNA was isolated and used for northern blotting (Figure 6) and RT-PCR (data not shown). Induction of c-myc RNA 1 h post serum addition was reduced approximately 40% in the HA-Rsu-1 transfectants compared to the control cells. However, c-Fos activation in the same filter was similar in HA-Rsu-1 transfectant and control cell lines. GPDH hybridization was used as a control for equal RNA loading in lanes. These results sugges-

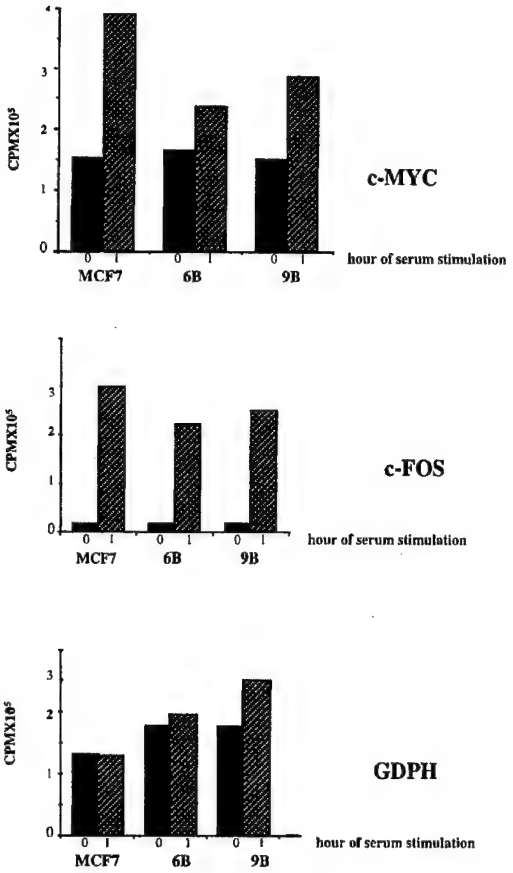


Figure 6. Expression of *c-myc* and *c-fos* in HA-Rsu-1 transfectants. The expression of *c-myc* RNA is response to serum stimulation was analyzed in HA-Rsu-1 transfectants and control cells. Cells were serum-starved for 16 h, then stimulated with 20% serum for 1 h. RNA was isolated and used for northern blotting. The same filter was probed for *c-myc* and *c-fos* and *GPDH* expression was used as a control for equal loading of RNA.

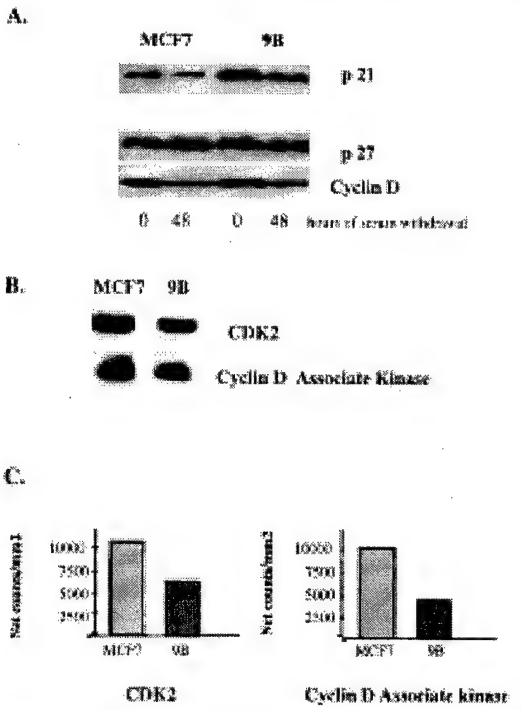


Figure 7. Cyclin dependent kinases and inhibitors in HA-Rsu-1 transfectants. A. The level of p21CIP, p27KIP, and cyclin D1 were determined by western blotting using lysates from cells grown in the presence of serum (10%) or from which serum had been removed (0.5%). The images were obtained from a single filter which was reacted separately with each antibody. B. Immune kinase assays were performed using lysates of cells grown in the presence of serum. The level of phosphorylation of GST-Rb substrate is shown. C. Quantitation of the amount of CDK2 and cyclin D associated kinase activity in the immune kinase assays shown in B.

ted that Rsu-1 had an inhibitory effect on *c-myc* RNA expression.

Expression of the cdk inhibitor p21^{CIP} in HA-Rsu-1 transfectant cell lines

Previous studies done in our laboratory indicated that expression of *Rsu-1* in PC12 cells resulted in an increase in p21^{CIP} levels [31]. An increase in the level of p21^{CIP} would contribute to a decrease in the growth rate of the transfectants due to potential for inhibition of cyclin D-cdk complex kinase activity. Therefore, the levels of p21^{CIP} were determined in HA-Rsu-1 MCF 7 transfectants and control cell lines. The amount of p21^{CIP} protein was determined by western blotting of total cell lysates obtained from cells growing in serum containing media or cells from which serum had been removed for 48 h. The results shown in Figure 7 indicate that the level of p21^{CIP} is clearly elevated in the HA-Rsu-1 transfectant cell line, 9B, compared to the control MCF7 cells and that the elevation is most evident under conditions of growth in serum. The levels of p27^{KIP} and cyclinD were determined by reaction of the same filter. The levels of p27^{KIP} and cyclinD were not affected by *Rsu-1* expression indicating, that the elevation of p21^{CIP} is specific [31]. In addition, there is an elevation in the amount of p53 protein in 9B cells compared to control cells (data not shown). Both cyclin D associated kinase activity and cdk2 activity were assayed by immune kinase reactions using the GST-Rb substrate and the results

indicated (Figure 7) that these activities were lower in the HA-Rsu-1 transfectant cell lysates containing elevated $p21^{CIP}$. These results indicated that the consequence of increased p53 and $p21^{CIP}$ was inhibition of G1 cdk activitiy.

Discussion

Our previous studies of cell lines expressing *Rsu-1* have indicated that elevated Rsu-1 levels are accompanied by activation of the ERK kinases, early response gene transcription and an inhibition of Jun kinase activity [30, 31]. In addition, Rsu-1 transfectant PC12 cell lines express elevated levels of $p21^{CIP}$compared to the control cell lines [31]. The results reported here extend our previous observation to MCF7 breast cancer cells in which the consequences of *Rsu-1* expression include an increase in p53 and $p21^{CIP}$and the inhibition of cdk2 and cyclin D associated kinase activities. Because Olson et al., have shown that the small GTPase Rho can function as a negative regulator of $p21^{CIP}$ levels [36] and because we have detected an inhibition of the Rho-dependent Rho kinase activity by *Rsu-1* expression, we suggest that a loss of Rho function in Rsu-1 transfectant cells may be the mechanism for the elevation in $p21^{CIP}$. There are other examples of inhibition of MCF7 proliferation by growth factor or inhibitor addition and by *HER* family expression with concommitant elevation of $p21^{CIP}$ [3, 9, 18, 44, 45]. In some cases it appears expression of $p21^{CIP}$ is p53-dependent because MCF7 cells contain wild type p53. However, it is not known if these cases of elevated $p21^{CIP}$ expression involve a change in Rho activity. Both tissue culture and *in vivo* results suggest that while a threshold level of $p21^{CIP}$ is required for formation of cyclin D1-cdk4 complexes and cell cycle progression, an increase in $p21^{CIP}$ can inhibit activity of cdks and alter their subcellular localization [21, 24]. While the level of cyclin D was not altered in HA-Rsu-1 transfectants, the change in levels of $p21^{CIP}$ would be expected to negatively regulate cyclin D associated kinase activity. This inhibition is highly relevant to breast cancer in part because cyclin D alterations are frequently observed in primary breast cancers [15, 40]. Therefore, the elevation of $p21^{CIP}$ and inhibition of cyclin D associated cdk activity as a result of *Rsu-1* expression is relevant to the study of growth inhibiton of breast cancer cells.

Tumor cells exhibit a number of different characteristics compared to their normal cell counterparts. An important phenotypic change is the loss of adhesion requirement for survival and growth under anchorage independent condition. HA-Rsu-1 transfectants exhibit a dramatic inhibition of growth in soft agar. Furthermore, the growth rates of Ha-Rsu-1 transfectants were slower than the control cell lines. We expect that this is a direct result of elevated $p21^{CIP}$ levels in the cells and reduction in cdk2 and cyclin D associated kinase activity. A study by Weaver et al., indicated that restoration of normal mammary cell phenotype to tumor cells and loss of anchorage independent growth potential by integrin blocking antibodies was accompanied by elevated $p21^{CIP}$ levels [42]. Also, another recent study demonstrated that Rho plays an important role in integrin regulation of adhesion in MCF7 cells [39]. Our previous study suggested that elevated *Rsu-1* expression altered regulation of Rho-dependent events (i.e. changes in actin cytoskeletal organization, inhibition of anchorage independent growth of epithelial cell) [30]. Therefore, decrease in a Rho function may be the relevant connection between the loss of anchorage independent growth and *Rsu-1* expression in the transfectant cell lines. Further studies will be needed to determine the role of Rsu-1 in the modulation of integrin expression and the mechanism of cellular adhesion relative to anchorage independent growth.

Analysis of the influence of *Rsu-1* expression on Ras-dependent pathways has indicated that Rsu-1 can exert its influence on multiple signaling pathways. Ras proteins are GDP/GTP-regulated switches that relay signals mediated by diverse extracellular stimuli to activate multiple effectors [1, 23, 43]. Activated Ras complexes promote the activation of Raf serine/threonine kinases, which then activate MAPK kinases (MEKs), which in turn activate the p42/p44 MAPKs. Our study showed that the activation of Erk-2 kinase induced by EGF was slightly increased in the Rsu-1 trasfectants than in control cell lines, confirming previous studies [30]. Also, the inhibition of Jun kinase activity relative to control cells was detected in breast cancer cell lines transfected with Rsu-1 suggesting that both Erk and Jun kinase pathway activation is indispensable for the induction of the transformed phenotype by Ras [38].

Our results show that induction of c-myc RNA 1 h post serum stimulation was reduced approximately 40% in the HA-Rsu-1 transfectants compared to the control cells. *c-myc* expression has since been shown to be altered in a wide variety of human tumors including breast, colon, and cervical carcinomas, small

cell lung carcinomas, osteosarcomas, glioblastomas, and myeloid leukemias (reviewed [16, 29]). The *c-myc* gene is involved in potentiation of cell cycle progression, inhibition of terminal differentiation, and induction of programmed cell death. The mechanism by which myc induces tumorigenesis is still poorly understood. The mechanism of downregulation of c-myc in the Rsu-1 clones has not been determined but may be related to changes in $p21^{CIP}$ level [6, 33]. Additional experiments will be necessary to further define the exact mode of control.

In summary, in MCF-7 Rsu-1 transfectant clones we found that Rsu-1 can exert its suppressor action in controlling multiple pathways which regulate biological properties, for example, anchorage independent growth. This study further emphasizes the complex nature of Ras-dependent signal transduction pathways in breast cancer and demonstrates that Rsu-1 mediates its suppressor actions through activation or inhibition of multiple signal transduction pathways in breast cancer cell lines.

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RSU-1 EXPRESSION RESULTS IN P53-DEPENDENT GROWTH INHIBITION IN FIBROBLASTS AND ENHANCES APOPTOSIS IN MCF7 CELL LINE

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Running Title: Rsu-1 enhances apoptosis in MCF7 cell line

ABSTRACT

Previous studies indicated that ectopic expression of the Ras suppressor Rsu-1 in the MCF7 breast cancer cell line inhibited anchorage independent growth which correlated with inhibition of Rho-dependent signaling events and CDK activity and an increase in the CDK-inhibitor $p21^{CIP}$. Additional staudies have shown that MCF7 Rsu-1 transfectants, which express wild type p53, exhibited an elevation of p53 compared to control cells, and pulse chase experiments determined that this was due to increased p53 protein stability. Activation of p53 by actinomycin D treatment of the cells resulted in two-fold increase in level of p53 in the Rsu-1 transfectants compared to control cells. Therefore, the effect of Rsu-1 expression on p53-dependent growth control was examined. Induction of apoptosis by TNFα and staurosporine is a p53-dependent event in the MCF7 cell line. Following treatment with staurosporine or TNFα plus cyclohexamide over a 14 hour time period the MCF7 Rsu-1 transfectant cell cultures contained nearly twice the number of apoptotic cells as the control cultures. Cleavage of PARP and MEK kinase proteins was also enhanced in Rsu-1 apoptotic cells compared to control MCF7. To investigate if the growth suppressive effects of Rsu-1 were dependent on p53, Rsu-1 was transfected into murine fibroblast cell line L929 in the presence and absence of expression of human papilloma virus E6 protein. The transfection of Rsu-1 significantly reduced colony formation by cells with functional p53 but not by cells in which p53 was inactivated. This result demonstrated that Rsu-1 growth suppression was p53-dependent in L929 cells. The contribution of Rsu-1 expression to p53 activation and p53-mediated apoptosis identifies Rsu-1 as a new target for the regulation of signaling pathways that mediate apoptosis.

INTRODUCTION

Rsu-1, initially identified as an inhibitor of Ras-induced transformation, can alter signal transduction downstream of Ras in multiple cell backgrounds (Masuelli & Cutler, 1996; Masuelli *et al.*, 1999; Vasaturo *et al.*, 2000). In MCF7 cell lines the expression of Rsu-1 inhibited anchorage independent growth, in part through inhibition of both Rho-dependent signaling events and CDK activity, the latter mediated by an increase of the level of the CDK-inhibitor $p21^{CIP}$ (Vasaturo *et al.*, 2000). $p21^{CIP}$ is transcriptionally regulated by p53 which functions as a transcription factor by binding to p53 consensus elements in a number of genes including the human ribosomal gene cluster (Kern *et al.*, 1991), muscle creatine kinase (Zambetti *et al.*, 1992), Mdm-2 (Wu *et al.*, 1993), GADD45 (Kastan *et al.*, 1992) as well as $p21^{CIP}$ (El-Deiry *et al.*, 1993). The p53 gene is one of the most frequently inactivated tumor suppressor genes in human tumors. Approximately 50% of all human cancers lack a wild-type p53 allele and/or produce a mutated version of the protein (as reviewed in (Hollstein *et al.*, 1994); (Hussain & Harris, 1998)). A variety of conditions can increase the activity of wild type p53, leading to the most important events mediated by p53, cell cycle arrest and apoptosis. Specific types of cellular stress, such as radiation, genotoxic chemicals, hypoxia, depletion of ribonucleotides, cause increased p53 protein levels through protein stabilization and induce p53-dependent biological response pathways (as reviewed in (Levine, 1997; Oren, 1999)). In addition, p53 activity is enhanced by a variety of oncogenic proteins, including Ras, Myc, adenovirus E1A, and β-catenin, activating the transcription of $p21^{CIP}$ among other responses [Debbas, 1993 #615; Hermeking, 1994 #614; Damalas, 1999 #616; Serrano, 1997 #455].

Regulation of $p21^{CIP}$ level is a function of the p53 checkpoint control for progression of the cell cycle from G1 to S phase. While $p21^{CIP}$ can induce growth arrest, p53 mediated G1 arrest can also be caused by a $p21^{CIP}$ independent pathway [Halevy, 1995 #585; Parker, 1995 #586; Zhang, 1995 #591]. The prognostic importance and predictive value of p53 has been studied in great detail in breast cancer, where inactivation of p53 is generally found in more advanced disease (Elledge & Allred, 1998). Inactivation of the p53 is associated with other known poor prognostic factors, including estrogen receptor (ER) negativity (Thorlacius *et al.*, 1993), progesterone receptor negativity (MacGrogan *et al.*, 1995), high proliferative fraction (Allred *et al.*, 1993), and poor nuclear/histologic grade (Thor *et al.*, 1992). Hence, alteration in regulation of p53-dependent events is relevant to etiology of breast cancer. Elevation of $p21^{CIP}$ by p53-dependent and independent mechanisms accompanies growth inhibition in breast cancer cell lines. For example, BRCA 1, a tumor suppressor gene mutated in hereditary breast and ovarian cancer (Miki *et al.*, 1994), contributes to cell cycle arrest and growth suppression though the induction of $p21^{CIP}$ in a p53-independent manner (Somasundaram *et al.*, 1997). However, Neu-differentiation factor-heregulin (NDF/HRG) and inactivating antibodies against erbB-2 induce growth arrest in breast cancer cells (Pietras *et al.*, 1994) through a p53-mediated pathway accompanied by upregulation of $p21^{CIP}$ (Bacus *et al.*, 1996).

Previous studies done in our laboratory indicated that expression of Rsu-1 in the MCF7 breast cancer cell line and the PC12 pheochromocytoma cell line resulted in an increase in $p21^{CIP}$ levels (Masuelli *et al.*, 1999; Vasaturo *et al.*, 2000). MCF7 cells contain wild type p53 and, therefore, in the current study the regulation of p53-dependent events by Rsu-1 was examined in the MCF7 cell line. To investigate the dependence of Rsu-1-induced growth inhibition on p53,

we introduced Rsu -1 into murine fibroblast L929 cell line in the presence and absence of human papilloma virus E6 protein, which initiates p53 degradation (Dbaibo *et al.*, 1998). The results reported in this study demonstrate an increase in p53-dependent apoptosis in Rsu-1 MCF7 transfectants as well as p53-dependent growth inhibition in L929 fibroblast cell lines in response to Rsu-1.

MATERIALS AND METHODS

Cell cultures and transfection. MCF7 cells and transfectants were maintained as described previously (Vasaturo *et al.*, 2000). L929 derived cell lines, L929-LXSN and L929-E6 were provided by Ghassan Dbaibo and Yusuf Hannun (Dbaibo *et al.*, 1998) and were maintained in Dulbecco Modified Eagle medium (DMEM) with 5% fetal bovine serum (FBS), and G418 (200 μg/ml). The L929 double transfectants, expressing either pZeoSV2 vector control (In Vitrogen) or pZeoSV2-HA-Rsu-1 (3V70), were selected following transfection using Lipofectamine 2000 (Life Technology) and maintained in G418 (200 μg/ml) and Zeocin (100 μg/ml).

Western blotting. Total cell lysates (100 μg) were isolated as described previously [Vasaturo, 2000 #550], separated by SDS-PAGE, transferred to nitrocellulose membrane and blocked in 1X blocking reagent: 2.5% nonfat dry milk in Tris-buffered saline. The filters were reacted with the different primary antibodies, washed in Tris-buffered saline containing 0.1% Tween 20 (Sigma), and incubated with specific horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science Inc., Arlington Heights). The filters were developed by enhanced chemiluminescence, (ECL) using Kodak X-Omat film. The primary antibodies used were: mouse anti-$p21^{CIP}$ (Pharmingen), mouse anti-p53 (DO-1) (Santa Cruz Biotechnology), rabbit anti-poly (ADP-ribose) polymerase (PARP) (Biomol Research Laboratories), mouse anti-Cyclin D1 (Santa Cruz Biotechnology), mouse anti-human Retinoblastoma protein (RB) (Pharmingen), mouse anti-$p27^{KIP}$ (Transduction Laboratories), rabbit anti MEK kinase (Santa Cruz Biotechnology) and rat anti-HA (Roche).

Pulse chase. The cell lines were preincubated in cysteine-methionine (cys-met) free DMEM containing 1% FBS and 10 mM Hepes for 30 minutes. The cell lines were labeled with cys-met free DMEM, 1% FBS, 20 mM Hepes and 0.5 mCi /ml ^{35}S met-cys Express Label (New England Nuclear) for 1 hour, then chase media was added and cells were harvested at appropriate times. The lysates were prepared in RIPA buffer and were precleared for 20 min. with fixed-staphylococcus A strain. The lysates were immunoprecipitated by adding 1 μg anti-p53 antibody (DO-1 SantaCruz) and protein A sepharose for 1 hour, and processing as described previously (Lee *et al.*, 2000b).

Colony formation assay. One μg of plasmid DNA containing Ha-Rsu-1, pZeoSV2-Rsu-1 (3v70), or pZeoSV2 vector control were transfected into L929 fibroblast cells expressing HPV E6 protein (E6) or L929 fibroblast vector control (LXSN) cells in 35 mm wells. 24 hour after transfection the cells were split to three 100 mm dishes, and were maintained in the G418- Zeo-containing medium for 20 days. At day 20 post transfection, cells were fixed and stained with Crystal Violet, and colonies were scored visually. The experiment was performed three times.

Cell death assay and treatment. 5 x 10^5 cells were seeded into 60 mm plates 48 hours prior to treatment. A combination of TNFα (15ng/ml) and cyclohexamide (10 μg/ml), or staurosporine (1 μM) was added to the cells in complete growth media. Detached cells were harvested and combined with the trypsinized attached cell population at specific time points after treatment. Cell death was assayed microscopically following addition of trypan blue. Living and dead cells were enumerated and additional evidence of apoptosis (nuclear condensation, membrane blebbing) was noted. Apoptosis was verified by assaying for cleavage of PARP and MEK kinase proteins

by Western blotting of total cell lysates. Lysates were prepared by resuspending cells in lysis buffer (0.5% Triton, 10% glycerol, 25 mM Hepes, 150 mM NaCl + protease inhibitors) from which samples were removed for determination of protein concentration. Lysates containing total cellular protein were mixed with 2X gel loading buffer containing 2% SDS and boiled for 5 minutes.

RESULTS

Rsu-1 expression results in elevation of $p21^{CIP}$ and p53 in MCF7 cell lines. Previous studies determined that ectopic Rsu-1 expression under the control of an inducible promotor in PC12 cells resulted in elevated levels of $p21^{CIP}$ but not $p27^{KIP}$ or cyclin D1 (Masuelli *et al.*, 1999) (Vasaturo *et al.*, 2000). However, in MCF7 Rsu-1 transfectants which constitutively expr4ess the transfected cDNA a more modest elevation of $p21^{CIP}$ was detected. In the present study MCF7 Rsu-1 transfectants were analyzed for levels of p53 protein to determine if the elevated $p21^{CIP}$ observed previously could be a result of change in the expression of p53 protein. The levels of $p21^{CIP}$ and p53 were assayed in total cell lysates of HA-Rsu-1 MCF 7 transfectants and control cells by Western blotting. The results shown in figure 1A indicate that the levels of both $p21^{CIP}$ and p53 are elevated in HA-Rsu-1 transfectant cell lines compared to the control MCF7 cells. However, as shown previously, levels of $p27^{KIP}$ and Cyclin D were unaltered by Rsu-1 expression.

Activation of p53 by actinomycin D treatment is enhanced by Rsu-1. Exposure of the MCF7 cell line to actinomycin D results in elevation of the level of p53 or p53 "activation" (Fang *et al.*, 1999). MCF7 control cells and MCF7-Rsu-1 transfectant cells were exposed to actinomycin D and then assayed for levels of p53 and hdm2. The results seen in figure 1B indicate that elevation of p53 in response to actinomycin D was observed in MCF7 control cells and elevation of p53 to higher levels was detected in MCF7-Rsu-1 transfectant cell line. Exposure to actinomycin D resulted in dramatically lower levels of hdm2 as expected due to disruption of p53-dependent activation of hdm2 transcription.

Rsu-1 expression enhances p53 stabilization. Changes observed in the level of p53 protein are generally due to an increase in p53 protein stability rather than an increase in p53 transcription (Oren, 1999). A pulse-chase experiment was used to determine if p53 upregulation in MCF7-Rsu-1 transfectants was due to altered stability of p53 (figure 2). Lysates were prepared from MCF7 control cells and the MCF7 Rsu-1 transfectants cells following a 60 minute labeling period and after 30 minutes and 90 minutes of chase. p53 was immunoprecipitated and detected by SDS-PAGE and autoradiography. The p53 protein was detectable in MCF7 control cell line at the conclusion of the labeling period but was undetectable by the end of the first chase period. In contrast, in MCF7 Rsu-1 transfectant clone 9B the p53 protein exhibited enhanced stability. Several p53 reactive bands were detected suggesting ubiquitination and turnover were underway, but not complete, at 30 minutes of chase (figure 2). This result indicated that p53 protein elevation in MCF7 Rsu-1 transfectants was due to increased p53 protein stability in this cell line. In addition, analysis of the expression of p53 RNA in the MCF7 and MCF7-Rsu-1 transfectant cell lines by quantitative RT-PCR determined that there was no change in level of p53 RNA in the Rsu-1 transfectants compared to the control cells (data not shown).

Rsu-1 inhibition of colony formation is a p53-dependent event. Rsu-1-dependent growth inhibition has been observed following ectopic expression of Rsu-1 in MCF7 breast cancer, NIH3T3 fibroblast, U251 glioblastoma and PC12 pheochromocytoma cell lines (Cutler *et al.*, 1992; Masuelli & Cutler, 1996; Masuelli *et al.*, 1999; Tsuda *et al.*, 1995). Following the determination that p53 was elevated in MCF7-Rsu-1 transfectants studies were initiated to determine if the growth suppressive effects of Rsu-1 were dependent on p53. We transfected Rsu-1 into murine fibroblast cell line L929 in the presence and absence of human papilloma virus

E6 protein (Dbaibo *et al.*, 1998). HPV-E6 and the cellular E6-AP form a complex and function as a ubiquitin ligase for p53 targeting it for destruction. Hence, this experiment was designed to test the effect of Rsu-1 expression in p53 active and inactive systems. Both the L929-HPV-E6 cell line and the L929-LXSN vector control cell line were transfected with pZeoSV2-Ha-Rsu-1 (3V70) or pZeoSV2 vector control, and the transfected cells were selected with Zeocin. At 18-20 days post-transfection the transfected cell cultures were stained and the extent of cell survival and cell growth was determined by recording the number and size of colonies which arose from the transfected cells. As reported in Table 1 colonies of Zeocin-resistant cells arose almost as frequently following transfection with the Rsu-1 vector as with the Zeo vector control in the L929-HPV-E6 (p53-inactivated) cell line. However, transfection of the pZeoSV2-Ha-Rsu-1 vector (3V70) resulted in a greatly reduced number of Zeocin resistant colonies compared to the pZeoSV2 control vector in the L929-LXSN vector control cell line, which contains wild type p53. Therefore, the relative efficiency of Zeocin-resistant colony formation by cells with functional p53 was significantly less in the presence of Rsu-1, but this effect was nearly eliminated by p53 inactivation. These results demonstrated that Rsu-1 exerted at least part of its growth suppressive effect in a p53-dependent manner. It should be noted that the vector used in this study contained an SV40 promoter to drive transcription of the Rsu-1 cDNA and the use of a stronger promoter might result in greater growth suppressive effects.

Apoptosis in MCF7 cells is enhanced by Rsu-1. Induction of apoptosis by TNF α or staurosporine is a p53-dependent event in MCF7 cell line (Amejar *et al.*, 1999; Cai *et al.*, 1997; Tang *et al.*, 2000). Because Rsu-1 expression stabilized p53 levels in MCF7 cells, the effect of

Rsu-1 expression on apoptosis was examined in the MCF7 background. The percentage of apoptotic cells was calculated following treatment of cells with TNFα (plus cyclohexamide) or staurosporine over a 14 hour time period, and the results, shown in Table 2A , demonstrated that the Rsu-1 transfectant cell lines exhibit a substantial increase in the number of apoptotic cells compared to MCF7 control cells. Initially the development of apoptosis was observed microscopically; nuclear condensation and membrane blebbing preceded cell rounding in treated cells. Apoptosis was scored in this experiment by trypan blue staining of treated and untreated cultures. The presence of nuclear condensation generally corresponded to typan blue uptake. This initial experiment revealed that the Rsu-1 transfectants cell line MCF7-9B was twice as sensitive to TNF α (plus cyclohexamide) and staurosporine than MCF7 control cell line (Table 2A). Subsequently the potential for an increased apoptotic response by other Rsu-1 transfectant cell lines was tested (Table 2B). The apoptotic response of the Rsu-1 transfectants was elevated compared to the control cells and paralled the level of HA-Rsu-1 expression in the individual transfectant cell lines.

Next biochemical markers of apoptosis were analyzed in MCF7 and MCF7-Rsu-1 transfectants. Two markers of apoptosis, cleavage of PARP (Duriez & Shah, 1997) and cleavage of Mek kinase (An & Dou, 1996; Janicke *et al.*, 1996; Tan *et al.*, 1997) were examined over 24 hours of induction. PARP cleavage and Mek kinase cleavage following TNFα or staurosporine treatments of MCF7 and MCF7-Rsu-1 transfectants were analyzed by Western Blotting using anti-PARP or anti-Mek kinase specific antibodies. The results, shown in Figure 3 along with the quantitation of phenotypic apoptosis, indicated that the biochemical analysis of apoptosis

corresponded to the microscopic analysis of the cells. Cleavage of PARP was illustrated by the disappearance of the p116/PARP and/or the appearance of the p85 PARP cleavage fragments. The cleavage of Mek kinase, documented by disappearance of the protein, was most apparent following 24 hours TNF α plus cyclohexamide treatment. Cleavage of both PARP and Mek kinase proteins was more apparent at times where higher levels of apoptosis, as indicated by typan blue exclusion staining, was seen. These results indicated that expression of Rsu-1 in MCF7 cells results in an enhancement of apoptosis in response to TNF α plus cyclohexamide or staurosporine.

DISCUSSION

Our previous studies of ectopic expression of Rsu-1 indicated that elevated Rsu-1 levels resulted in an increase in $p21^{CIP}$ in MCF7 and PC12 cell lines (Masuelli *et al.*, 1999; Vasaturo *et al.*, 2000). Furthermore, transfection of Rsu-1 inhibited anchorage-dependent and -independent growth of U251 glioblastoma and MCF7 tumor cell lines (Tsuda *et al.*, 1995; Vasaturo *et al.*, 2000). The growth inhibitory effects of Rsu-1 expression in MCF7 cells were correlated with alterations in signal transduction pathways through Rho, cyclin D-associated kinase activity, CDK2, and the CDK inhibitor $p21^{CIP}$. The present study demonstrated that Rsu-1 transfectants in MCF7 cells, which contain wild-type p53, exhibit increased stability of p53. Moreover, expression of Rsu-1 in MCF7 cells, with subsequent stabilization of p53, resulted in an increase in p53-dependent apoptosis in response to both TNFα and staurosporine. The p53-dependent effects of Rsu-1 on growth were not limited to MCF7 breast cancer cells. Using p53 positive and negative L929 fibroblasts the dependence of Rsu-1 growth inhibition on p53 was observed. It is likely that the upregulation of p53 is responsible for elevated expression of the CDK inhibitor $p21^{CIP}$. While other mechanisms of action may yet be elucidated, our present results indicate that the p53-dependent events, including induction of $p21^{CIP}$ and enhanced apoptosis, may be critical for the growth inhibition induced by Rsu-1.

The mechanism by which Rsu-1 expression stabilizes p53 is not clear. The induction of p53 is primarily regulated at the level of protein stability as a result of altered MDM2 binding (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). Degradation of p53 is dependent on the E3 ligase activity of MDM2 (Haupt *et al.*, 1997; Honda *et al.*, 1997; Kubbutat *et al.*, 1997) and nuclear

export of p53 and MDM2. (Roth *et al.*, 1998) (Lohrum *et al.*, 2000). The up-regulation of p53 by oncogenes, including Ras, may be dependent on the presence of the $p19^{ARF}$ protein and its interaction with HDM2 by blocking HDM2 ubiquitin ligase activity (Honda & Yasuda, 1999; Kamijo *et al.*, 1998; Pomerantz *et al.*, 1998; Stott *et al.*, 1998; Zhang *et al.*, 1998). The human homolog of $p19^{ARF}$, $p14^{ARF}$, physically interacts with MDM2 and blocks MDM2-dependent p53 degradation when introduced into MCF7 cells (Midgley *et al.*, 2000; Midgley & Lane, 1997). However, because MCF7 cells express little or no endogenous $p14^{ARF}$ (Stott *et al.*, 1998) it is unlikely that Rsu-1 expression stabilizes p53 dependent activation via a $p14^{ARF}$ dependent step. Studies by Ries et al. (Ries *et al.*, 2000) demonstarted that mdm2 expression can be induced by activation of Ras or Raf and that in the absence of $p19^{ARF}$ this results in p53 down regulation. This mechanism of p53 regulation may be compromised by Rsu-1 inhibition of Ras activity. Another mechanism by which p53 is stabilized involves its phosphorylation. Phosphorylation of p53 on serine 20 enhances its stabilization by inhibition of MDM2 binding resulting in nuclear accumulation of p53 (Aschroft *et al.*, 2000). ATM and ATR kinases, which are activated in response to DNA damage phosphorylate p53 on serine 15, a site not required for p53 stabilization but which is involved in the apoptotic response. Erk and p38 kinase have also been reported to phosphorylate this site. In addition, ATM and ATR activate the Chk1 and 2 serine/threonine kinases which phosphorylate p53 on serine 20 triggering dissociation of p53-MDM2 complex, and phosphorylation of p53 on serine 46 appears to regulate transcriptional activation of apoptosis-inducing genes (Oda *et al.*, 2000). Enhanced p53 serine 20 phosphorylation in the Rsu-1 transfectant clones could account for changes in p53 stability. Previous studies of Rsu-1 transfectants demonstrated elevated Erk activity which could be an

important link between Rsu-1-induced cellular change and p53 stability, and future studies will address this issue.

$p21^{CIP}$ expression is transcriptionally regulated by p53 both constitutively and in response to DNA damage (Tang *et al.*, 2000). The mechanism by which Rsu-1 inhibits growth is likely linked to upregulation of p53 resulting in elevated $p21^{CIP}$ and inhibition of pRb phosphorylation (Vasaturo *et al.*, 2000) as well as to the increased sensitivity of Rsu-1 transfectant cell lines to apoptosis. Recent studies in the MCF7 cell line demonstrated that TNFα-induced apoptosis was dependent upon wild type p53 expression (Amejar *et al.*, 1999; Cai *et al.*, 1997). Moreover, the sensitivity of MCF7 cells to TNFα correlated with elevated expression of $p21^{CIP}$ and MDM2, down regulation of c-myc and degradation of Rb (Amejar *et al.*, 1999). The results of our study are in good agreement with this report. While the data on expression of c-myc in Rsu-1 transfectants was not presented here, a previous report demonstrated that c-myc expression was reduced in both dividing and growth factor stimulated MCF7-Rsu-1 transfectants when compared to control cells (Vasaturo *et al.*, 2000). Also, neither our study (M.L. Cutler, unpublished data) nor that of Ameyer observed a change in Bcl2 expression following TNFα treatment of the cells. Staurosporine-induced apoptosis shared many of the same properties as TNFα-induced apoptosis in our study.

Under some circumstances, activation of the Ras pathway arrests the cell cycle via activation of the Raf/ERK MAP kinase pathway through the upregulation of p53 and, in part, the induction of $p21^{CIP}$ (Serrano *et al.*, 1997). Ras activation of Rho-dependent signal transduction downregulates $p21^{CIP}$ allowing cell cycle progression (Olson *et al.*, 1998). In this

publication we show that Rsu-1 expression which upregulated Erk activity and inhibited Rho activity, altered p53 levels and induced the cyclin-dependent-kinase inhibitor $p21^{CIP}$. Therefore, the induction of p53 and $p21^{CIP}$ by Rsu-1 may be regulated through an activation of MAPK and inhibition of Rho signaling. Alternatively, stabilization of p53 by Rsu-1 may explain the activation of the Ras-Erk pathway in MCF7-Rsu-1 transfectants (Lee *et al.*, 2000a). Rsu-1 stabilization of p53 provides a probable mechanism for Rsu-1 in contributing to p53-mediated apoptosis. Identifying the contribution of Rsu-1 to p53 activation and p53-mediated apoptosis focuses on Rsu-1 as a component of a signaling pathways that mediates p53-dependent apoptosis and growth arrest.

Figure Legends

Figure 1. **A.** Expression of p53 and $p21^{CIP}$ in Rsu-1 MCF7 transfectants. Western blots of cell lysates from MCF7 and MCF7-Rsu-1 transfectant cell lines reacted with p53, $p21^{CIP}$, $p27^{KIP}$ and cyclin D antibodies. p53 and cyclin D separated on a 10% gel; HA-Rsu-1, $p21^{CIP}$ and $p27^{KIP}$ separated on a 12% gel. 100 μg of protein loaded per lane. Lane 1, MCF7; lane 2, MCF7-Rsu-1-6B; lane 3, MCF7-Rsu-1-9B; lane 4, MCF7-Rsu-1-14B. **B.** Actinomycin D treatment of MCF7 transfectant. MCF7 and MCF7-Rsu-1-9B cell lines were exposed to actinomycin D (100μg/ml) for 16 hours. Western blot of total cell lysates separated by SDS-PAGE and reacted with anti-p53 and anti-mdm2 antibodies. Lane 1, MCF7 t=0; lane 2, MCF7 t=16 hours; lane 3, MCF7-Rsu-1-9B t=0; lane 4, MCF7-Rsu-1-9B t=16 hours.

Figure 2. Pulse-chase labeling of p53 in MCF7 and MCF7-Rsu-1 transfectant cell lines. Lysates were prepared from ^{35}S pulse labeled cells at 0, 30 and 90 minutes after the addition of the chase media. p53 was immunoprecipitated from lysates and separated by SDS-PAGE. Auto-radiography of gel is shown and the position of immunoprecipitated p53 is indicated by an arrow.

Figure 3. Cleavage of PARP, Rb, and Mek kinase proteins in MCF7 and MCF7 Rsu-1 transfectant cells following apoptosis. Total cell lysates were prepared from MCF7 and MCF7-Rsu-1-9B cells untreated cells and those exposed to staurosporine (1μg/ml) for 4 or 8 hours and

TNFα (30 ng/ml plus 10 μg/ml cyclohexamide) for 8 or 24 hours. **A**. Western blots of the lysates separated on 8% SDS-PAGE were reacted with anti-PARP antibody and detected with enhanced chemiluminescence. **B**. Western blots of the lysates separated on 8% SDS-PAGE were reacted with anti-Rb kinase antibody and detected with enhanced chemiluminescence. **C**. Western blots of the lysates separated on 8% SDS-PAGE were reacted with anti-Mek kinase antibody and detected with enhanced chemiluminescence.

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Table 1

Colony formation following transfection of Rsu-1 into L929 fibroblasts.

		Transfection #1 [a]		Transfection #2 [a]	
Cell Line	**Vector**	**CFU**	**% of pZeo SV2**	**CFU**	**%of pZeoSV2**
LXSN	pZeo SV	384	(100)	520	(100)
LXSN	p3V70	230	(60)	180	(34)
E6	pZeo SV	567	(100)	380	(100)
E6	p3V70	526	(93)	275	(72)

a. transfection of L929-LXSN and L929-HPVE6 cell lines was performed as described in Materials and Methods. CFU= colony forming units per microgram of plasmid DNA in each experiment.

Table 2

A. Apoptosis in MCF7 control and MCF7 –Rsu-1-9B transfectants following TNFα or staurosporine treatment.

Cell line	Treatment [a]	% apoptotic cells [b]
MCF7-control	(-)	< 0.1
	Stauro 14 hours	48
	TNF+CHX 14 hours	40
MCF7-Rsu-1-9B	(-)	<0.1
	Stauro 14 hours	90
	TNF+CHX 14 hours	81

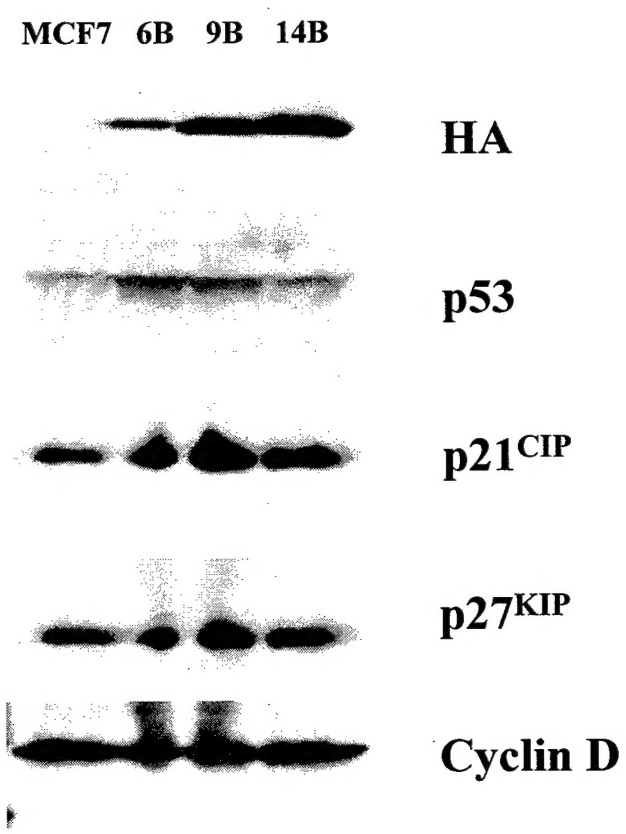
B. Apoptosis in MCF7 and 6B, 9B, 14B Rsu-1 transfectants following staurosporine treatment.

Cell line	Treatment [a]	% apoptotic cells[b]
MCF7-control	(-)	<0.1
	Stauro 16 hours	35
MCF7-Rsu-1-6B	(-)	<0.1
	Stauro 16 hours	51
MCF7-Rsu-1-9B	(-)	<0.1
	Stauro 16 hours	64.3
MCF7-Rsu-1-14B	(-)	<0.1
	Stauro 16 hours	46

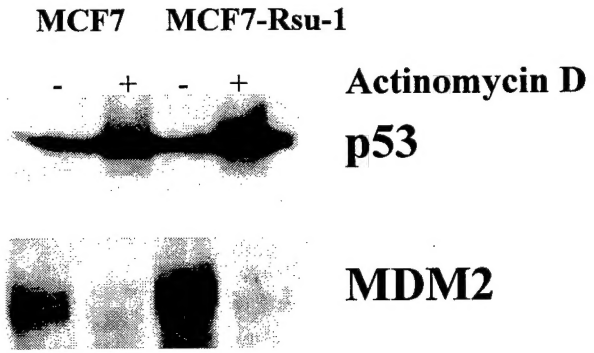
a. 5×10^5 cells were seeded into 60 mm plates and exposed to Staurosporine (1μM) or TNFα (15 ng/ml) plus cyclohexamide (10 μg/ml) for time indicated as described in Materials and Methods.

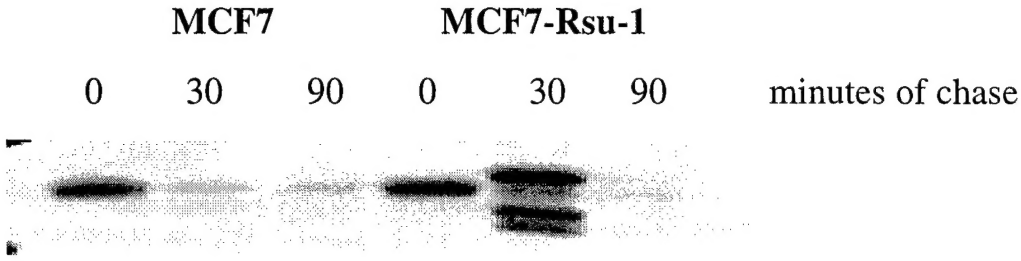
b. Apoptosis was determined by microscopic evaluation of trypan blue stained cells as described in Material and Methods. % of apoptotic cells was calculated by dividing number of dead cells by total number of cells for each sample. Data in B was derived from 4 experiments, statistical analysis by T test, $p < 0.001$.

A.

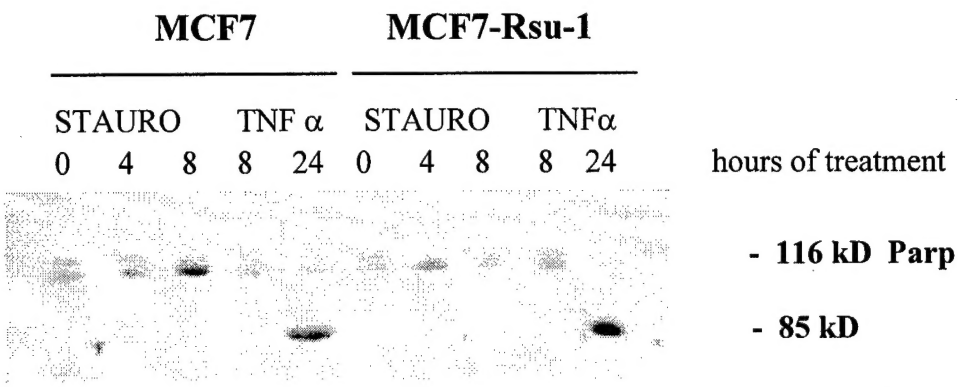

MCF7 6B 9B 14B
HA
p53
p21CIP
p27KIP
Cyclin D

B.

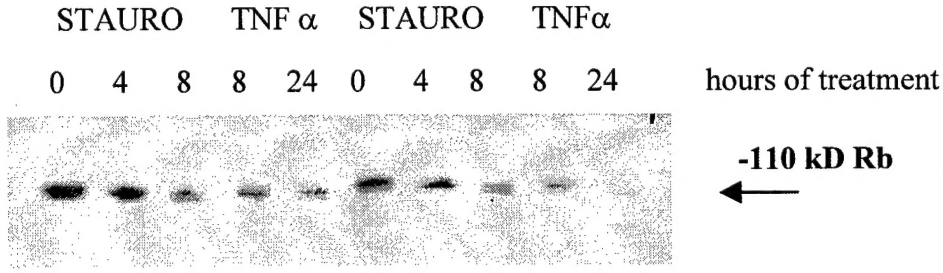

MCF7 MCF7-Rsu-1
- + - +
Actinomycin D
p53
MDM2


MCF7
MCF7-Rsu-1
0
30
90
0
30
90
minutes of chase

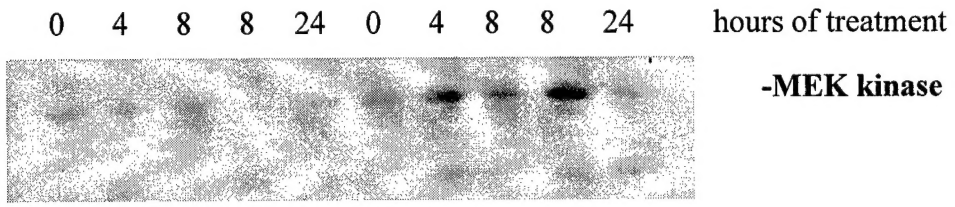
A


MCF7
MCF7-Rsu-1
STAURO
TNF α
STAURO
TNFα
0 4 8 8 24 0 4 8 8 24
hours of treatment
- 116 kD Parp
- 85 kD

B


STAURO
TNF α
STAURO
TNFα
0 4 8 8 24 0 4 8 8 24
hours of treatment
-110 kD Rb

C


0 4 8 8 24 0 4 8 8 24
hours of treatment
-MEK kinase